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Part IV

Environmental Protection Agency

Proposed Health Effects Test Standards for Toxic Substances Control Act Test Rules and Proposed Good Laboratory Practice Standards for Health Effects

ENVIRONMENTAL PROTECTION AGENCY

[FRL 1279-6; OTS-046003B]

[40 CFR Parts 770, 771, 772]

Proposed Health Effects Test Standards for Toxic Substances Control Act Test Rules; Proposed Good Laboratory Practice Standards for Health Effects; Extension of Comment Period and Rescheduling of Public Meeting

AGENCY: Office of Toxic Substances, Environmental Protection Agency (EPA).

ACTION: Notice extending the time period for comments on a notice of proposed rulemaking and rescheduling a public meeting.

SUMMARY: This notice extends the time period for the submission of comments on two proposed rulemaking actions: (1) the proposed Health Effects Test Standards for Toxic Substances Control Act Test Rules (Chronic Effects Test Standards) and (2) the proposed Good Laboratory Practice Standards for Health Effects. The comment period is extended from August 7, 1979, to October 16, 1979. This notice also reschedules public meetings on the two proposals for October 15 and 16 in Chicago. The extension and the rescheduling are made on the initiative of the Agency so that the end of the comment period and the date of the public meeting will coincide.

DATE: Interested persons are invited to submit written comments to the EPA on the proposed test standards prior to the close of business on or before October 16, 1979. The public meetings on the two proposals are rescheduled for October 15 and 16 in Chicago.

ADDRESSES: Written views and comment should bear the document control number OTS No. 046003B and should be submitted to: Document Control Officer, Office of Toxic Substances (TS-793), EPA, 401 M Street, SW., Washington, D.C. 20460.

FOR FURTHER INFORMATION CONTACT: Industry Assistance Office, Office of Toxic Substances (TS-799), EPA, 401 M Street, SW., Washington, D.C. 20460, Telephone No.: 800-424-9065 (toll free). In Washington, D.C. call 554-1404.

SUPPLEMENTARY INFORMATION: On May 9, 1979 (44 FR 27334), the EPA issued a notice of proposed rulemaking proposing regulations on: (1) the Health Effects Test Standards for Toxic Substances Control Act Test Rules (Chronic Effects Test Standards) and (2) the Good Laboratory Practice Standards for Health Effects (40 CFR Parts 770-772). Interested persons were invited to submit comments to the EPA on the proposed test standards prior to the close of business on or before August 7, 1979.

In the same notice of proposed rulemaking, the EPA announced public meetings on the proposed test standards to be held during the week of July 9 in Chicago and during the week of July 16 in Washington, D.C. Subsequently, the EPA announced in the issue of June 28, 1979 (44 FR 37682) the postponement of the public meetings. The meetings have now been rescheduled for October 15 and 16 in Chicago.

Consequently, the Agency has determined that the close of the comment period on the proposed test standards should coincide with the dates of the public meeting in Chicago. Therefore, the deadline for the submission of comments to the EPA on the proposed test standards is extended to the close of business on or before October 16, 1979.

Dated: July 16, 1979.

Steven Jellinek,

Assistant Administrator for Toxic Substances.

[FR Doc. 79-22848 Filed 7-25-79; 8:45 am]

BILLING CODE 6560-01-M

[40 CFR Part 772]

[FRL 1269-7; OTS 046005]

Office of Toxic Substances; Proposed Health Effects Test Standards for Toxic Substances Control Act Test Rules

AGENCY: Environmental Protection Agency.

ACTION: Proposed rule and notice of public meetings.

SUMMARY: This action proposes test standards for the development of health effects data on chemical substances and mixtures for which testing will be required under the Toxic Substances Control Act (TSCA). Standards are proposed for the testing of acute and subchronic toxicity, mutagenic effects, teratogenic/reproductive effects, and metabolism studies. The Environmental Protection Agency (EPA) will codify these proposed standards for incorporation into future chemical specific TSCA Section 4 test rules. In addition, the Agency may also use these standards to respond to Section 4(g) petitions for test standards for new chemical substances.

EPA proposes these test standards under Section 4 of TSCA. It is intended that data from these proposed standards will be utilized by EPA to determine whether the tested chemicals present an unreasonable risk to human health and may be used to support regulatory actions to eliminate or reduce such a risk.

The test methods proposed here are substantially similar to the methods proposed by EPA for its pesticide registration program. See the "Proposed Guidelines for Registering Pesticides in the U.S.; Hazard Evaluation: Humans and Domestic Animals" (43 FR 37336, August 22, 1978).

DATES: Interested persons are invited to submit written comments to EPA on these proposed test standards no later than the close of business on or before October 16, 1979. EPA has extended the comment period for its May 9, 1979 proposal on Chronic Effects Test Standards and Good Laboratory Practices (44 FR 27334 *et seq.*) to end on the same date as given above for this proposal. EPA has scheduled a public meeting on this proposal as well as the proposal in the Chronic Effects and the Good Laboratory Practice Standards for Health Effects published May 9, 1979 (44 FR 27334 *et seq.*). This meeting will be held on October 15 and 16, 1979, in Chicago. See Section VIII, Public Meetings, for further information.

ADDRESSES: Written views and comments should bear the document control number OTS No. 046005 and should be submitted to: Document Control Officer, Office of Toxic Substances (TS-793), Environmental Protection Agency, 401 M Street, S.W., Washington, D.C. 20460.

PUBLIC MEETINGS: EPA plans to hold a two-day public meeting on this proposal and the proposal on Chronic Effects and Good Laboratory Practices published on May 9, 1979 (44 FR 27334 *et seq.*) on October 15 and 16, 1979 in Chicago. The public meeting will be held at: Holiday Inn—Chicago West, 1900 North Mannheim Street, Melrose Park, Illinois 60160. (EPA has canceled the previously announced public meetings on the May 9 Chronic Effects proposal and related GLP so that all the health effects test standards may be considered together in October's public meeting.) Additional information on this meeting is presented in Section VIII, "Public Meetings."

FOR FURTHER INFORMATION CONTACT: Industry Assistance Office, Office of Toxic Substances (TS-799), Environmental Protection Agency, 401 M Street, S.W., Washington, D.C. 20460,

Telephone No. 800–424–9065 (toll-free), (In Washington, D.C., call 554–1404).

SUPPLEMENTARY INFORMATION: The following is an index to the remainder of this preamble.

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I. Introduction

In the May 9, 1979, **Federal Register**, EPA proposed under Section 4 of the Toxic Substances Control Act (TSCA) (Pub. L. 94–469; 90 Stat. 2006; 15 U.S.C. 2603) standards for the development of data on chronic health effects (44 FR 27334 *et seq.*). With the proposal, EPA initiated the process to codify health effects standards for incorporation into future TSCA Section 4 test rules. Today, EPA is continuing the process of codifying test standards by proposing standards for testing chemicals for acute and subchronic toxicity, mutagenic effects, teratogenic effects, reproductive effects, and metabolism studies.

Later this year, the Agency will propose the first chemicals to be tested in accordance with Section 4 test standards. In the present rulemaking to codify test standards, the issues center on the suitability of the proposed generic standards for testing chemicals under future Section 4 test rules. In later rulemakings to require the testing of specific chemicals or categories of chemicals, the issues will center on the need to test the identified chemicals.

When EPA determines that chemical substances satisfy the testing prerequisites in Section 4(a), these chemical substances will be joined in Section 4(a) test rules with the standards finalized as a result of today's proposal, or with revisions or modifications of these standards, as necessary and appropriate.

EPA's legal authority to require testing of chemical substances in accordance with test standards and the use of test data for assessing the risk potential of toxic substances are set forth in the May 9, 1979, **Federal Register** notice proposing Chronic Health Effects Standards (44 FR 27335–36).

II. Background

According to the May 9, 1979, **Federal Register** notice, new Parts 770, 771, and 772 would be added to Chapter I of Title 40 of the Code of Federal Regulations. Proposed Part 770—"Test Rules for Chemical Substances and Mixtures"—provides information and other general regulatory criteria applicable to all health and environmental effects test rules. Part 771—"Identification of Chemical Substances and Mixtures to be Tested"—will identify the specific chemical substances to be tested using certain specific test standards when chemical specific test rules are promulgated. Part 772—"Standards for the Development of Test Data"—states the test methods to be used when a manufacturer or processor is required to test those chemicals that will be listed under Part 771.

The May 9 notice proposed two of the Part 772 subparts. Subpart B—"Good Laboratory Practice Standards" (44 FR 27362)—proposes general standards for conducting animal bioassay laboratory health effects studies. Subpart D—"Chronic Health Effects" (44 FR 27350–27362)—proposes standards for oncogenic effects, non-oncogenic chronic effects, and combined oncogenic/non-oncogenic chronic effects. Today, EPA is proposing additional subparts: Subpart A (General Requirements); Subpart C (Acute and Subchronic Health Effects), Subpart E (Mutagenic Effects); Subpart F (Teratogenic/Reproductive Effects), and Subpart I (Other Health Effects—Metabolism).

III. Consistency With Pesticide Guidelines

In the notice proposing the chronic effects test standards, EPA stated its policy of reducing the burden on the regulated public that might arise from conflicting requirements under different sets of test regulations and guidelines that may be promulgated under different Federal authorities (44 FR 27344). By this proposal, the Agency continues to implement this policy by proposing test standards under TSCA regulations substantially similar to EPA's guidelines for testing required to support pesticide registration under the Federal Insecticide, Fungicide, and Rodenticide Act ("FIFRA"); 7 U.S.C., Section 136 *et seq.*

EPA's Office of Pesticide Programs has proposed test standards which contain data requirements comparable to those proposed under TSCA test standards. The proposed FIFRA rules are published at 43 FR 37336 (August 22, 1978); the comment period closed on November 20, 1978, but is being reopened to solicit additional comments. A separate notice in the **Federal Register** will indicate the date that will reopen the FIFRA comment period.

TSCA and FIFRA may differ with respect to when a test must be performed, however, EPA has decided that the standards for performing that test should be the same under either authority, to the extent permitted by different statutory requirements. Accordingly, EPA is proposing under TSCA health effects test methods and data reporting requirements for acute and subchronic effects, teratogenic/reproductive effects, mutagenic effects, and metabolism studies similar to those which EPA proposed under FIFRA in August, 1978, and is incorporating in this proceeding the public record in the FIFRA proceeding.

Note.—Other health effects test standards (e.g. neurotoxicity) are being developed and will be proposed in future TSCA test rule proceedings.

Since the organizations of the TSCA and FIFRA standards differ, it would be useful to briefly summarize the different standards to facilitate understanding of how the various sections relate. FIFRA has proposed human hazard evaluation test rules as Subpart F of Part 163, volume 40 of the Code of Federal Regulations (40 CFR, Part 163). Section 163.80 sets forth general requirements for human hazard testing. These requirements include general personnel qualifications and other general good laboratory practice standards for the conduct of laboratory studies, including animal care standards, necropsy procedures, tissues, and slide preparation. The FIFRA rules also set forth general reporting requirements in § 163.80–4.

Subsequent sections of Part 163, Subpart F deal with specific test methods—acute (Section series 163.81), subchronic (Section series 163.82), chronic including oncogenic (Section series 163.83), teratogenic/reproductive (Section series 163.83), mutagenic (Section series 163.84), and general metabolism (Section series 163.85). The sections describing each test generally begin with a paragraph entitled "When required," which establishes the conditions under which the particular test is needed to support pesticide registration. A paragraph entitled "Standards" states the acceptable methods for performing the test. These standards cover such aspects of test methodology as the substance to be tested and its condition; the selection of test species; the age, sex and number of test animals; the route and duration of exposure; the number and selection of dose levels; clinical testing and observation; and necropsy and histopathology as they apply to specific

tests. A paragraph describing the data reporting requirement for each specific test follows the "Standards" paragraph.

The TSCA health effects standards are organized differently to some extent as a result of the different statutory requirements of the separate acts. Part 770 contains provisions that relate to the authority of EPA under TSCA, such as general compliance requirements, confidentiality provisions, and general definitions. Part 771, which will contain the chemicals to be tested under TSCA, will serve a purpose similar to the "when required" paragraph of the FIFRA test rules. Part 771 will identify the substance to be tested (after EPA makes the findings required by Section 4(a) of TSCA), will provide deadlines for submission of data, and prescribe standards for the development of data by reference to the test standards in Part 772.

Part 772, which will identify acceptable test standards for development of data, will be consistent with the comparable provisions of the FIFRA test rules. As currently proposed, however, there are basic organizational differences in the TSCA and FIFRA test rules. Where the FIFRA good laboratory practice standards for all health effects testing under FIFRA are generally set forth in § 163.80–3 and –4 (43 FR 37352–55) and in a new proposal § 163.80–6 and –7, the TSCA good laboratory practice standards may be found in two different parts of the TSCA standards. First, Subpart A states basic requirements for personnel, general pathology, test substance concentration, dietary considerations, and contaminant analysis. Second, the TSCA Good Laboratory Practice Standards section (Subpart B, Part 772) contains general provisions on such matters as conduct of laboratory studies, animal care, and personnel requirements that will apply to all health effects testing.

Any differences between FIFRA and TSCA good laboratory practice requirements will be resolved before either regulation is finalized.

At the heart of the TSCA standards are the specific sections describing the required methods to be used for each test. These sections are comparable to the "Standard" paragraph of the FIFRA test rule sections, and deal with such factors as selection of species, sex, age and number of test animals; route and duration of exposure; number and selection of dose levels; clinical testing and observation; and test specific requirements for necropsy and histopathology. These are the factors that will govern how a test is to be performed. In this notice, EPA proposes for its TSCA standards the basic test methods (with some modifications) proposed in August 1978 for the equivalent FIFRA test standards. In addition, EPA proposes as part of its TSCA standards the same data reporting requirements as the FIFRA rules.

All persons commenting on these proposed TSCA test standards should carefully review the FIFRA proposed rules and comment on the applicability of the FIFRA standards for use as test standards under TSCA. Any of the proposed FIFRA methods may be adopted as TSCA test standards following Agency review of the public comments from both the FIFRA and TSCA proceedings.

While the TSCA test methods proposed today are in many respects the same as the comparable FIFRA methods, EPA believes that it should solicit additional comments on issues as they relate to the proposed tests. These additional issues will be referenced in this notice as they apply to the specific test.

IV. Basis and Purpose

The primary purpose of toxicological studies is to determine the adverse effects of chemicals on biological systems and to acquire data on dose-response characteristics of the test system following exposure. These data may then provide information relating to risks to human health or the environment posed by a chemical substance.

Toxicity testing can be divided into two major categories—short-term and long-term testing. The major differences between these studies, in addition to duration of exposure, is the number and range of dose levels employed. In general, more dose levels and a greater dose range are employed in short-term studies in order to estimate the dose-response relationship of the chemical.

Acute toxicity studies are designed to determine the toxic effects of exposure to a chemical over a short time period. These studies generally provide data to determine the median lethal dose (LD_{50}) of a chemical substance (its relative toxicity), but also may provide data to approximate its mode(s) of action, to determine its specific toxic effect(s) on target organs and functions, and to determine the existence and extent of species differences in sensitivity to a chemical.

Subchronic toxicity studies consist of repeated exposure (usually daily) to a chemical substance over a period of approximately 90 days. These data provide information on the major toxic effects of test substances and target organs or functions that may be affected following multiple doses. These studies may also provide information on the delayed effects of chemicals that may be due to bioaccumulation of test substances or their metabolites. The reversibility of these effects as well as the kinetic characteristics of the chemicals may also be studied. Subchronic test information is of fundamental importance in designing long-term studies in which exposure may extend for several months or years.

The major objective of teratogenic and reproductive testing is to identify chemical substances that may affect the production of offspring and the normal development of the fetus and offspring. The thalidomide tragedy of the early 1960's clearly indicates the importance of knowing the impact of chemicals on the developing fetus and its eventual survival. This proposal is intended to provide standards for the development of data indicating effects on reproductive organs and general reproductive performance, fertilization, implantation, toxic effects on the pregnant mother, the embryonic period (tissue differentiation and organogenesis), the fetal period (prenatal growth and functional maturation), birth, lactation and maternal care of the offspring, and postnatal growth, survival, and fecundity.

In addition to the Agency's concerns for the normal development of the fetus and newborn offspring, the impact of chemicals on future generations is of fundamental importance. The chemical substance may have its effect upon the genetic material within the nucleus of the cell(s) of either the reproductive organs of the male (sperm) and/or female (eggs), upon the genetic material of developing or maturing offsping, or upon the somatic cells of the test species. In order to evaluate these types of effects, the Agency will be proposing that certain mutagenic tests be performed with specific chemical substances. It is intended that these mutagenic tests will provide information relating to whether a chemical substance may present a risk or causing genetic damage to humans. At present, no definitive method is available to directly measure mutagenic potential in humans. Therefore, mammalian, nonmammalian, and microbial systems are used to provide information to evaluate the mutagenic hazard to humans caused by exposure to particular chemicals.

EPA also recognizes that selected mutagenic tests have potential benefit in

concogenic screening and that the scientific community is actively assessing the results of both *in vitro* and *in vivo* bioassay methods. This issue need not be elaborated in this proceeding, however, since EPA's decision concerning the circumstances under which mutagenic tests will be required will properly be the issue in proceedings on rules that designate specific chemcials for testing under Part 771 of the TSCA regulations. The public will be provided adequate opportunity to comment should the Agency consider whether certain of the proposed mutagenic test methods should be used for oncogenic screening.

The Office of Testing and Evaluation in EPA is assessing the current status of tests in genetic toxicology through its Gene-Tox program. Panels of experts are reviewing the literature on a number of mutagenic test systems, including the thirteen test standards listed in the FIFRA rules and proposed in this proceeding. As a part of this evaluation, the experts are considering the significance of the previous test results within each system, the adequacy of the test methods, and will recommend the most efficient test battery to detect potential mutagenic effects. The deliberations of the Gene-Tox program will be considered in the final rulemaking proceedings. In addition, scientific review processes will be employed in arriving at final standards. These deliberations including those of the Gene-Tox program will be taken into consideration as a part of the final rulemaking proceedings.

These metabolism studies are designed to develop information on the disposition of a test substance within the test system. In the case of these health effects studies, the test system would be at least one mammalian species, preferably the rat. However, more than one species would be desirable, in particular, other species anticipated to be used in long-term studies. More specifically, these metabolism studies would provide information on how the test substance is absorbed and transported within the test system, how it changes form including transformation into other chemicals (i.e. metabolites), or how and to what extent it accumulates in the test species. These studies are of particular importance in selecting test species for long-term studies. The bioaccumulation of the test substance or its metabolites may well impact upon the long-term, low level exposure(s) and the subsequent toxicity of the test substance.

Some confusion has been expressed with respect to the use of the term "metabolism" studies in the FIFRA proposal. This issue has been raised on the basis that the objectives of the standards proposed are concerned with the kinetic properties of the test substance in the biological test system. The World Health Organization is considering the use of the term "chemobiokinetic" to describe these types of kinetic studies whereas some scientists have suggested the term "toxicokinetic". The Agency invites public comment on the appropriate term to be used to describe these types of studies.

V. Proposed Test Standards

General

A detailed discussion of the major scientific issues involved in this proposal is presented in the preamble to the FIFRA proposal (43 FR 37336). Issues discussed in this preamble to the TSCA proposal are those required because EPA believes that further consideration and comment are needed. This section of the preamble outlines the proposed TSCA test standards and briefly discusses the additional scientific issues involved.

EPA is proposing as standards for development of test data under TSCA the basic test methods (with some modifications) proposed by the Agency's Office of Pesticide Programs under FIFRA. The public is invited to submit comments on the applicability of using the specifically referenced FIFRA standards as TSCA standards. The Office of Testing and Evaluation (OTE) will be working closely with the Office of Pesticide Programs (OPP) to harmonize their respective standards so that they may be published as final rules that are as consistent as possible in form and content as permitted by both statutes.

Those who have already commented on the proposed FIFRA rules need not resubmit those same comments on this proposal because all public comments will be reviewed by both OTE and OPP before final publication of test rules. However, further additional comments are invited.

Although the proposed TSCA and FIFRA test methods and reporting requirements are generally the same, EPA notes below issues associated with certain test methods that may result in further change in test rules. The public is also specifically invited to comment on any of the issues discussed, as well as any other issues that deal with alternative test methods. Comments should be as specific as possible and state reasons why one alternative is preferable to another. Appropriate references should be included.

Summary of Standards

Subpart A—General Requirements

Section 772.100–1 General.—This section describes the general scope and purpose of the test standards in Part 772 and states that the applicability of these standards will be specified in Part 771 of this subchapter.

Section 772.100–2 General Standards for Health Effects.—This section describes general requirements that are applicable to all health effects studies. These requirements include the basic standards for specific personnel, submission of study plans when specified, test or control substance concentration, dietary requirements when specified (i.e., chronic, reproductive, and teratogenic studies), contaminant analysis of feeds and/or vehicles, clinical and general pathology procedures, and general reporting requirements. These general requirements are referenced in standards on specific effects in subsequent subparts. The specific effects standards in the other subparts also will specify more detailed test standards on the same subject when appropriate.

When the specific test standards are promulgated in the near future for Ecological Effects and Chemical Fate, Subpart A will be amended to include those additional General Requirements.

In addition, several of the General Requirements now proposed in Subpart A have been previously proposed in Subpart D for the Chronic Effects Standards (44 FR 27334–27362). When Subpart D is promulgated as a final rule, those general requirements will be deleted from Subpart D and listed in Subpart A.

This TSCA proposal differs from the FIFRA proposal in that it sets forth specific qualifications for certain scientific and support personnel, whereas EPA has previously proposed these specific personnel requirements in its TSCA Chronic Effects Test Standards (see 44 FR 27388, 27350–51). EPA requests comment on which qualifications are appropriate for personnel conducting tests under TSCA and FIFRA.

The TSCA proposal also proposes the same general data reporting requirements for acute and subchronic, mutagenic, teratogenic, reproductive, and metabolism studies as contained in the proposed FIFRA rules (§ 163.80–4; 43

FR 37354–55). However, the Agency is considering a common format for all test data. EPA is participating with other members of the Interagency Regulatory Liaison Group (IRLG) in developing a common government-wide data format. EPA will consider comments on any of the various data reporting schemes proposed under TSCA or FIFRA or any alternative method for developing data format provisions.

The TSCA proposal also requires detailed analyses of test and control substances (§ 772.100–2(b)(3) of Subpart A). The same section was proposed as part of the TSCA Chronic Effects Test Standards (§ 772.113–1(g); 44 FR 27338–9, 27351). The FIFRA proposal lists the analytical requirements for test substances in § 163.80–3 under the title "Test Substance", in § 163.80–4 under "Substance tested", and in Subpart D, chemistry requirements, § 163.61–6.

The general histopathology methods and other general clinical procedures described in the TSCA proposal for acute and subchronic tests as well as those proposed for teratogenic and reproductive tests are those proposed for the TSCA Chronic Effects Test Standards (§ 772.113–3(b) (1) and (2); 44 FR 27357–27359). These standards are more detailed in their description of how to carry out certain procedures such as gross necropsy than those in the FIFRA proposal (§ 163.80–3(b) (1) and (11); *also see* FIFRA sections on individual tests, for example, §§ 163.81 and 163.82).

The extent of necropsy and histopathology that should be conducted for various types of studies has been the subject of considerable discussion. This proposal, as well as the FIFRA proposal, requires rather extensive procedures even for acute toxicity studies because the Agency believes that these procedures are necessary to identify reliably the potential toxic effects of a chemical. Microscopic tissue examination, even for acute studies, may be necessary to reveal valuable information about toxic effects which would not be observable through observation of the whole animal. A more detailed discussion of the Agency's position on this issue is contained in the preambles to the FIFRA rules and the TSCA Chronic Effects Test Standards. (See 43 FR 37343–44; 44 FR 27343.)

The proposed TSCA test standards for health effects testing state that observations of animals must be made to insure that losses in any test group due to cannibalism, autolysis of tissues, misplacement, or similar management problems do not jeopardize the validity of the study. In this regard, losses must not exceed 5 percent for groups of animals greater than 20 per group and a loss no greater than one (1) animal per group for group sizes less than 20. (*See* Subpart A, § 772.100–2(b)(6)). In addition, the Agency proposes that animals must be observed at least every 12 hours. The loss of animals due to management problems are less likely under these provisions. The limits of loss (e.g. 5%) and frequency of observation are more stringent than proposed in the FIFRA rule, which requires a 10% loss limit and observations at least once each morning and late afternoon thereafter. The public is invited to comment on the appropriateness of these quality control procedures.

Subpart C—Acute and Subchronic Health Effects

Section 772.112–10 General.—This section includes requirements and definitions generally applicable to acute and subchronic health effects testing. Acute effects are defined as short-term health effects following single administration of a test substance. Subchronic studies are defined as "health effects following continuous or repeated administration of a test substance over a period of approximately 90 days."

The acute effects following a single exposure to a test substance will be utilized to estimate the hazard from accidental test substance exposure, to provide information relating to handling and labeling of the test substance, and to provide information for subchronic studies. The acute studies also will be utilized to estimate the relative toxicity and lethality of the test substance. In addition, observation will be made of the physical appearance and behavioral syndromes of toxicity.

The subchronic effects data will be utilized to estimate the hazard associated with multiple, short-term exposures of the test substance. These subchronic studies will provide additional information to help evaluate kinetic characteristics of the test substance and will provide information on the impact of the test substance on various functional systems within the host. This information also will be utilized to estimate anticipated effects of chronic exposures.

The principle sections of Subpart C are the test methods sections for individual tests.

The following list summarizes the section and title of the test standard and the corresponding test standard from the FIFRA proposal.

Section of TSCA proposal	Test standards	Section of FIFRA proposal
772.112–21	Acute oral toxicity study	163.81–1(b) and (c).
772.112–22	Acute dermal toxicity study	163.81–2(c) and (d).
772.112–23	Acute inhalation toxicity study	163.81–3(b) and (c).
772.112–24	Primary eye irritation study	163.81–4(c) and (d).
772.112–25	Primary dermal irritation study	163.81–5(c) and (d).
772.112–26	Dermal sensitization study	163.81–6(b) and (c).
772.112–31	subchronic oral dosing studies	163.82–1(c) and (d).
772.112–32	Subchronic 90-day dermal toxicity study	163.82–3(c) and (d).
772.112–33	Subchronic inhalation toxicity study	163.82–4(c) and (d).

EPA requests comment on all aspects of the acute and subchronic test methods as set forth in this notice and the proposed FIFRA rules. There are several issues concerning the conduct of testing on which EPA requests comment, as follows:

(1) *Age and Number of Species.* Scientific opinion varies with respect to the age and number of species that should be required in acute studies. The proposed FIFRA guidelines, and the standards proposed in this notice, require only one species and age at the start of the study. The Agency, however, recognizes that increasing the number of species and age range tested would decrease the possibility of missing a potentially toxic effect due to variability in the susceptibility of various species and ages to a given test substance. In addition, increasing the number of species and age range would increase the confidence in which one could extrapolate the toxic potential to humans. Two ages and two species are being considered for acute effects testing for the final test rules. Any comments on this issue should specifically compare the relative benefits of using one or more species and more than one age and include appropriate scientific references.

(2) *Histopathology in Acute Studies.* Scientific opinion also varies with respect to the use and extent of histopathology in acute studies. The Agency recognizes that histopathology would be desirable in all hazard

assessment studies but is also sensitive to the additional costs imposed by such requirements. However, EPA is considering histopathology on all observable changes found at gross necropsy in acute oral studies. In addition, the final test standards for acute dermal studies (§ 772.112–22(b)(5) of Subpart C) may require histopathology of all tissues with gross changes in addition to the skin. The use and the selection of appropriate tissues to be weighed at gross necropsy as a measure of organ specific toxicity is also a scientific question posed in acute and subchronic studies. The Agency may modify the number and types of tissues to be weighed in these studies as a result of public comment and further assessment by the Agency. The public is invited to comment on these potential changes and is requested to provide the supporting scientific rationale for all recommendations submitted.

(3) *Exposure Period.* The Agency is considering at least a 4-hour exposure period in acute inhalation studies (§ 772.112–23(a)(3)). The FIFRA proposal requires at least a 1-hour exposure period but may require up to 4 hours (§ 163.81–3(b)(4); 43 FR 37357). It may be that only potent chemicals would be effective with only a 1-hour exposure period and, therefore, this requirement may be modified. In addition, the use of pathogen-free animals for inhalation studies is an issue that is being considered for the final rule. The use of these animals would help clarify the effects of the test substance without compromising the histopathology. The public is invited to comment further on these issues and to submit appropriate scientific rationale and data.

(4) *Subchronic Studies.* One of the major issues that is of concern to the Agency relates to the appropriate use of 90-day, subchronic studies in nonrodents (e.g. dog). The Agency has proposed that preliminary toxicology studies of at least 90 days are required to select the chronic dose levels for long-term studies (see 44 FR 27357 and 27360). If the intent of the study is only for purposes of selecting chronic dose levels, the Agency may consider using subchronic data from 90-day studies. However, if the intent of the study is to evaluate the data for hazard assessment purposes, then the 6-month study is required.

(5) *Starting Age of Nonrodent.* The TSCA and FIFRA proposals require that non-rodents be started on study at 4–6 months of age. The Agency also intends to use the subchronic data from the non-rodent as guidance for long-term studies and has proposed a 10-week starting age for the dog in the requirements as stated in the proposed Chronic Effects Test Standards (44 FR 27334 *et seq.*). The Agency recognizes that the starting age of the nonrodent (e.g. the dog) at 10 weeks of age may present certain problems such as obtaining weaned animals and acclimatizing them appropriately before the initiation of the study. It has also been suggested that blood chemistry and hematology measurements will be somewhat variable in the younger animals. The Agency requests comment including supporting scientific data as to the appropriate age to start the dog when attempting to meet the needs of evaluating subchronic data for hazard assessment purposes as well as guidance for long-term chronic studies.

(6) *Extent of Histopathology.* The selection of tissues in subchronic studies for histopathologic examination also may be more extensive in the final TSCA and FIFRA rules. The public is encouraged to comment on the present list of tissues for histopathologic examination including the procedural approach described for setting priorities in the evaluation of rodent tissues for histologic examination according to test group (*See* §§ 772.112–31, 772.112–32, and 772.112–33).

Subpart E—Mutagenic Effects.

Section 772.114–1 General.

The Agency is concerned with the potential of certain chemical substances to cause mutations in humans and other species. A detailed discussion of this potential health problem can be found in the preamble to the FIFRA Guidelines (43 FR 37347–37349, August 22, 1978) and in a paper entitled "Criteria for Evaluating the Mutagenicity of Chemicals" ("Criteria Paper"). The latter document was published as Addendum III to the FIFRA proposed rule.

The Agency proposes as TSCA test standards the proposed FIFRA mutagenic test methods including the general discussion in § 163.84–1(a), (d), (e), and (f) of the FIFRA proposal (43 FR 37388–89).

The following list summarizes the corresponding mutagenic effects standards from the proposed FIFRA rules and those proposed as a part of these proceedings under TSCA.

Subsection of TSCA proposal	Test standard	Subsection of FIFRA proposals
772.114–1	General requirements for mutagenic tests	163.84–1(a)(d)(e)(f)
772.114–2	Test standards for detecting gene mutation	163.84–2
772.114–3	Test standards for heritable chromosomal mutations	163.84–3
772.114–4	Test standards for detecting effects in DNA repair of recombination as an indicator of genetic damage.	163.84–4

As a part of the annual review of test methods under TSCA, the current status of the mutagenic effects test standards will be reviewed and amended as appropriate. The public is invited to comment on these test methods.

Subpart F—Teratogenic/Reproductive Health Effects

Section 772.116–1 General.—This section deals with the scope and purpose as well as the applicability of teratogenic and reproductive studies. These health effects data will be used to assess the hazard of a test substance on the production of offspring and on the normal development of the fetus and offspring. More specifically, the teratogenic studies will provide information on the potential of a test substance to produce defects in progeny (offspring) resulting from exposure during gestation. Reproductive effects studies will provide hazard assessment information resulting in an impairment of reproduction due to test substance exposure.

The test standards for both teratogenic fand reproductive effects tests reference the general requirements for personnel, chemical analyses, dietary consideration, clinical observations, pathology procedures, and general reporting requirements in Subpart A of this subchapter (see Section 772.100–2).

In addition, a Study Plan is proposed in these standards for reproductive effects studies only. The Study Plan must be submitted to EPA ninety days prior to the initiation of the reproductive effects studies. Submission of this Study Plan to the Agency does *not* mean that EPA intends to approve or disapprove the Study Plan. The Study Plan must contain the information as stated in Section 772.100–2(b)(2), Subpart A. The Agency believes that acute and subchronic studies as well as teratogenic, mutagenic and metabolic studies are of a short-term nature and review of these procedures can best be

handled following submission of the test results to the Agency.

It is also proposed that Summary Reports be submitted on a quarterly basis for reproductive studies which would provide a current status of the study. It is expected that these interim summary reports be brief (5–10 pages) and provide summary information on significant findings of such parameters as survival, weight changes, clinical test results, accumulative incidence to developmental alterations and toxicity.

Section 772.116–2 Teratogenic Test Standards.—The proposed TSCA teratogenic standards are adopted from § 163.83–3 (b) and (c), *Teratogenicity Studies*, of the FIFRA proposal (43 FR 37382–84). Comments should be directed to all issues raised in the FIFRA record. EPA requests additional comments on the following issues related to the conduct of teratogenic studies:

(1) EPA is considering requiring reporting of individual live fetal weights, rather than only requiring the average live fetal weight, in order to determine unusual patterns of reduced maturity.

(2) The number of rabbits in each test and control group may be increased from 12 to at least 20, the same number used for rat, mouse or hamster tests.

(3) More extensive soft tissue or skeletal examinations of fetuses may be required.

(4) The inclusion of data from animals which show 100 percent fetal death may be required.

Section 772.116–3 Reproductive Effects Test Standards.—The Agency proposes to adopt § 163.84–4 (b) and (c), *Reproduction Study*, of the FIFRA proposal as TSCA Section 4 Standards.

EPA requests comment on all issues on reproductive effects studies raised in the proceeding on the FIFRA guidelines. The Agency notes that certain additional issues are being considered with respect to conduct of reproductive effects studies:

(1) Should the number of males and females in each dose group be equal in order to generate equal statistical evaluation for males and females? The proposal requires 10 males per dose group and enough females to produce 20 litters. *See* A. K. Palmer. "Some thoughts of reproductive studies for safety evaluation." Toxicology: Review and Prospect. Proceedings of the European Society for the Study of Drug Toxicity, vol. XIV, p. 82. Excerpta Medica International Congress Series No. 288.

(2) The proposal requires dosing of the first two generations of a three generation study. EPA is considering dosing only the first generation in order to determine the effect on the second generation of *in utero* exposure to the test substance. Dosing of the second generation as it matures may result in confusing the *in utero* effects with the effects of direct dosing.

(3) EPA is considering whether to use a "split study" technique whereby half the second generation is sacrificed near term and the other half sacrificed after weaning. The sacrifice near term allows better assessment of fertility and while implantation sites are still present. An estimate of embryo and fetal wastage can also be made. In the remaining half, general performance relating to reproduction and pathology from *in utero* exposure and lactation can be assessed. This split study may necessitate a greater number of animals be used in the reproductive effects study.

(4) EPA may require under certain circumstances, more extensive examination of test animals including offspring.

Subpart I. Other Health Effects

Section 772.119–1 General Metabolism Test Standards.—The Agency proposes as TSCA test standards the test methods stated in the *General Metabolism Studies* from the FIFRA proposal (§ 163.85–1 (a), (c), (d), and (e); 43 FR 37394–96).

In general, these proposed standards may be used to estimate the extent to which the test substance and/or its metabolites are absorbed, distributed, and bioaccumulated. This information will be used in the assessment of species selection, route of administration, and dose selection rationale for long-term studies.

During the development of the pesticide standards, considerable discussion focused on the issue of the appropriate number of species to be used in metabolism studies. The economic and scientific factors involved in using more than one species for metabolism studies still remains an open issue. The public is invited to submit further comment on this issue.

Request for Comments on Proposed Test Standards

In the previous paragraphs of this preamble, several issues of scientific concern have been raised. The public is invited to submit comments and supporting scientific data on any issue in the proposed standards. Comments on the following issues are of particular interest to the Agency:

(a) Qualification of professional personnel conducting these tests,

(b) Choice of species including sex and status of the females (e.g. nonpregnant, nulliporous),

(c) The appropriate number of test animals per group necessary to make adequate and reliable comparisons,

(d) The age and weight of the animals at the start of the study,

(e) Number of dose levels, and the rationale for dose selection necessary to achieve adequate exposure and dose-response information,

(f) The extent and detail of fasting before dosing,

(g) The selection of tissues for gross and microscopic pathology including the extent of the microscopic evaluation,

(h) The selection of appropriate mutagenic tests,

(i) The frequency of weight measurements during each study.

VI. Economic Analysis

A regulatory analysis is not required with respect to these proposed standards because the standards themselves do not impose any costs on any person at this time. Rather, these standards are being developed as part of an array of standards to be used selectively as needed. EPA believes, however, that the proposed standards are necessary to assure that data developed through test rules are adequate for their intended use in hazard and risk assessments. Abbreviated test standards could result in a significant reduction in the quality and reliability of the resulting data and could preclude adequate assessment of hazard potential.

The estimated cost per chemical of each test (unit cost) required by the FIFRA rules was analyzed by the Office of Pesticides Programs in a report entitled "Economic Impact Analysis of Proposed Guidelines for Registering Pesticides in the United States" (43 FR 39664, September 6, 1978, Table 12). The Agency has received a few comments concerning these estimated unit costs. The OPP cost analysis and the comments received on these costs are available upon request from the Industry Assistance Office at the address listed at the beginning of this preamble under "For Further Information Contact". The Agency invites comment on the OPP estimates and the subsequent comments on these estimates.

Because of the substantial similarity between the TSCA health effects standards proposed today and the proposed FIFRA standards, the OPP cost analysis and the subsequent comments are used as the basis for this proposal. The Agency intends to review the FIFRA cost estimates when it prepares

the cost analysis on the final health effects test standards. Therefore, EPA requests comment on the following issues:

(1) The difference in unit cost between the proposed TSCA and FIFRA standards, since these two have differences in personnel requirements, Good Laboratory Practice requirements, and other requirements.

(2) The extent to which the FIFRA estimates require revision and/or updating.

(3) The increase or decrease in unit cost resulting from any modifications to the standards discussed in this preamble, and

(4) The increase or decrease in unit cost resulting from any modifications to the standards suggested by the commentor.

VII. Public Participation

During the development of these standards for proposal as FIFRA Guidelines, several public meetings were held. A detailed discussion and listings of these meetings are cited in the Preamble to the FIFRA proposal (43 FR 37336, August 22, 1978).

Note.—Comments previously presented to the Agency in response to the proposed Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) Guidelines for the Registration of Pesticides (43 FR 37336, August 22, 1978), will be reviewed along with any comments to this proposal in finalizing the TSCA Standards. Previous submitters to the FIFRA proposal may supplement their earlier comments with respect to the FIFRA Guidelines as referenced in this proposal as appropriate requirements for Section 4 of TSCA.

VIII. Public Meetings

EPA plans to hold a two-day public meeting at the Holiday Inn-Chicago West (Melrose Park, Illinois) on October 15 and 16, 1979. This meeting will address both this proposal and the proposal of May 9, 1979 on the Chronic Health Effects and Good Laboratory Practice Standards for Health Effects (44 FR 27334 *et seq.*). The public meetings mentioned in the May 9 proposal have been canceled by EPA to allow all the Section 4 health effects standards to be addressed together. The written comment period for the earlier proposal has been extended to end on the same date as does this proposal's, October 16, 1979.

The purpose of this public meeting is to enable interested persons to provide oral comments in the proposed rulemaking to EPA officials who are directly responsible for developing the standards in this rulemaking. All remarks and presentations will be transcribed by EPA and entered on the public record. EPA officials responsible for developing these test standards will conduct the meeting and use the following format. The moderator will recognize people from the floor who wish to comment, discuss or clarify issues in both proposals; he will call for remarks on each part and subpart in its order of appearance in this combined rulemaking, viz., Part 770, Part 772, Subparts A through F and I.

IX. Public Record

EPA has established a public record for his rulemaking (docket number OTS 046005) which, along with a complete index, is available for inspection in the OTS Reading Room from 9:00 a.m. to 5:00 p.m. on working days (Room 447E, 401 M Street, S.W., Washington, D.C. 20460.) This record includes basic information considered by the Agency in developing this proposal. The Agency will supplement the record with additional information as it is received. The record includes the following categories of information:

(1) US EPA–OTS. "Proposed Health Effects Test Standards for Toxic Substances Control Act Test Rules" (44 FR 27334 *et seq.*).

(2) US EPA–OPP. "Proposed Guidelines for Registering Pesticides in the United States; Hazard Evaluation: Humans and Domestic Animals" (43 FR 37336 *et seq.*), and all public comments received on this proposal.

(3) US EPA–OTS. "Proposed Good Laboratory Practice Standards for Health Effects" (44 FR 27369 *et seq.*)

(4) US EPA–OPP. "Economic Impact Analysis and Reports Impact Analysis of Guidelines for Registering Pesticides in the United States" (43 FR 39644 *et seq.*).

(5) Minutes, summaries, or transcripts relating to public meetings held to develop the FIFRA and TSCA standards.

(6) Correspondence and public comments between outside persons and EPA personnel pertaining to development of the FIFRA and TSCA standards. (This does not include any inter- or intra-Agency memoranda unless specifically noted on the index of the rulemaking record).

(7) Scientific documents supporting and/or relating to the scientific issues raised in this proceeding.

Published documents cited in any document in this record are incorporated in the record by reference. The record of the proceedings to establish guidelines for human and domestic animal hazard evaluation under FIFRA is incorporated in the record in this proceeding by reference (FIFRA docket no. 30023). EPA will accept additional material for the record at any time between this proposal and the final designation of the rulemaking record. EPA will identifying the complete rulemaking record on or before the date of promulgation of these requirements, as prescribed by TSCA Section 19(a)(3). The final rule will also permit persons to point out any errors or omissions in the record. The record of this proceeding is available in the OTS Reading Room.

Note.—Under Executive Order 12044, EPA is required to judge whether a regulation is "significant" and therefore subject to the procedural requirements of the Order or whether it may follow other specialized development procedures. EPA labels these other regulations "specialized." This regulation has been reviewed and it has been determined that it is a specialized regulation not subject to the procedural requirements of Executive Order 12044.

(Section 4, Toxic Substances Control (TSCA) (90 Stat. 2006; 15 U.S.C. 2603).)

Dated: July 13, 1979.

Douglas M. Costle,

Administrator, Environmental Protection Agency.

On May 9, 1979, at 44 FR 27334, EPA proposed to add a new Part 772 to title 40 of the Code of Federal Regulations consisting of Subparts B and D. The remaining subparts of proposed Part 772 were reserved. In this document EPA proposes to add Subparts A, C, E, F, and I to the proposed Part 772 to read as set forth below.

PART 772—STANDARDS FOR DEVELOPMENT OF TEST DATA

Subpart A—General Requirements

Sec.
772.100–1 General.
772.100–2 General standards for health effects.
Appendix A.
Appendix B—Dietary requirements and contaminant analysis.

* * * * *

Subpart C—Acute and Subchronic Health Effects

772.112–10 General.
772.112–21 Acute oral toxicity study.
772.112–22 Acute dermal toxicity study.
772.112–23 Acute inhalation toxicity study.
772.112–24 Primary eye irritation study.
772.112–25 Primary dermal irritation study.
772.112–26 Dermal sensitization study.
772.112–31 Subchronic oral dosing studies.
772.112–32 Subchronic 90-day dermal toxicity study.
772.112–33 Subchronic inhalation toxicity study.

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Subpart E—Mutagenic Effects

Sec.
772.114–1 General.
772.114–2 Test standards for detecting gene mutations.
772.114–3 Test standards for detecting heritable chromosomal mutations.
772.114–4 Test standards for detecting effects on DNA repair or recombination as an indicator of genetic damage.

Subpart F—Tetratogenic/Reproductive Health Effects

772.116–1 General.
772.116–2 Teratogenic effects test standards.
772.116–3 Reproductive effects test standards.

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Subpart I—Other Health Effects

772.119–1 General metabolism test standards.

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Authority: Section 4; Toxic Substances Control (TSCA), 90 Stat. 2006; 15 U.S.C. 2603.

Subpart A—General Requirements

§ 772.100–1 General.

(a) *Scope and purpose.* The standards in this subpart are designed to provide general test requirements for health and environmental effects test standards subject to test regulations under the Toxic Substance Control Act (TSCA) (Pub. L. 94–469, 90 Stat. 2003, 15 U.S.C. 2601 *et seq.*). The EPA will use these data and information to assess the risk chemicals may present to human health and the environment.

(b) *Applicability.* These standards apply to the development of health and environmental effects data from the testing of chemicals specified in part 771 of this subchapter.

§ 772.100–2 General standards for health effects.

(a) *Scope.* The standards contained in this section apply to all health effects studies in Section 772. Specific subsections of Section 772 contain complementary and additional requirements that are test standard specific on the same subject. In such a case, the specific standards in the subsection apply in addition to those specified in this subpart.

(b) *Basic standards for testing.*—(1) *Specific personnel requirements.* For the purposes of these test standards:

(i) There are two types of qualified pathologists:

(A) Board-Certified or Board Eligible pathologist(s) who have a minimum of three years of experience in pathology of the species of laboratory animals to be used; or

(B) Other doctorate pathologists (D.V.M., M.D., Ph. D., D.D.S., D.O.) who have a minimum of three years of experience in gross, clinical, and/or histopathology of the species of laboratory animals to be used.

(ii) A qualified veterinarian is one who is Board-Certified or eligible for certification by the American College of Laboratory Animal Medicine, and who has a minimum of two years of experience in laboratory animal science; and

(iii) There are two types of qualified technical employees:

(A) One certified by the American Society of Clinical Pathology (HTASCP), or with equivalent training and experience.

(B) One certified or eligible for certification by the American Association of Laboratory Animal Science (LTAALAS), or with equivalent training and experience.

(2) *Submission of study plan.* (i) When required by a specific test standard, the sponsor(s) of a study must submit a detailed study plan to the Agency at least 90 days before the study is initiated.

(ii) The following information must be submitted:

(A) The name and address of the sponsor and testing facility including responsible administrative officials and project manager(s);

(B) Brief summaries of the training and experience of each professional involved in the study, including Study Director, Veterinarian, Toxicologist(s), Pathologist(s) and Pathology Assistants;

(C) Study protocol including rationale for species strain selection, dose selection (and supporting data), route(s) of exposure, modifications or variance from applicable TSCA standards and rules;

(D) Summary of expected spontaneous chronic diseases (including tumors), genealogy, and lifespan of the test species;

(E) Description of diet to be used and source of diet;

(F) Data on test substance and stability under test and storage conditions;

(G) Schedule for intition and completion of major phases of long-term(s); schedule for submission of interim progress and final reports to EPA.

(3) *Test or control substance concentration.* When required by the applicable test standard, the tester must document that, at the time of administration, the test substance or mixture administered contains no less than 90 percent of the designated test substance concentration as specified in the sponsor-approved protocol. To accomplish this objective, the tester must conduct and document stability studies in accordance with Subpart B, § 772.110–1, prior to initiation of a study and analyze the administered substance or mixture to determine:

(i) The rate of loss of the test substance by elution, degradation, or other means;

(ii) The major degradation products, if any; and

(iii) Analyze for initial concentration.

The initial mean concentration of the test substance must not vary more than ±5 percent from the designated concentration. To assure homogeneity in a test mixture, variability among randomly selected samples (at least 3) from the test mixture must not exceed ±10 percent from the mean of the samples.

Note.—If a vehicle is needed in the preparation of the test mixture, either to dissolve, dilute or otherwise facilitate mixing or administering the test substance, the vehicle should be selected to have the following characteristics:

(1) It facilitates absorption but does not substantially alter the extent of distribution, metabolism, or retention of the test substance;

It does not alter the chemical properties of the test substance or substantially enhance, reduce, or alter the toxic characteristics of the test substance;

(3) It does not substantially affect the food or water consumption or the nutritional status of the animals;

(4) It does not produce substantial physiological effects at the levels used in the study.

(4) *Dietary requirements.* (i) When required by the applicable test standards, all rodents must be fed the standardized diet containing the nutrient levels and produced by feed stocks or ingredients as listed in Appendix B of this section unless EPA approves another diet that the sponsor proposes as a substitute. The sponsor must request and obtain EPA approval at the time of study plan submission (*see* paragraph (b)(2)) for diets for non-rodent species.

(ii) The tester must not use feed 90 days after its manufacture. The tester must maintain as a part of the raw data a log showing the date each batch/lot of feed was manufactured and the last date it was administered.

(5) *Contaminant analysis of feeds and/or vehicles.* The sponsor must have each batch/lot of feed and vehicle analyzed to determine concentrations of contaminants listed in Appendix B of this section. The Agency considers this list to be minimal and not all inclusive. The Agency encourages the tester to

conduct additional analyses for other contaminants that may affect the interpretation of the study results.

(6) *Clinical procedures.* (i) A veterinarian, as specified in § 772.100–2 (b)(1)(ii), must ascertain and be responsible for the health status and care of all test animals during the study. A technical employee, as specified in §772.100–2 (b)(1)(iii)(B), must be responsible for the daily observations and care of the test animals.

(ii) *Observation of animals.* (A) Each test animal must be identified by a specific identification number. The tester must account for all animals at the end of the study. The tester must establish and adhere to standard operating procedures for housing, feeding, handling, and care of test animals as specified in § 772.110–1. To further assure the development of valid data, observation of animals must be made by a qualified technical employee or by a qualified professional scientist (e.g. veterinarian or toxicologist) at least every 12 hours throughout the test period to ensure that losses in any test group due to cannibalism, autolysis of tissues, misplacement, or similar management problems do not exceed 5 percent for groups of animals greater than 20 per group and a loss no greater than one (1) animal per group for group sizes less than 20. EPA may consider a study to be unacceptable for purposes of satisfying a test rule requirement if losses do not meet these requirements.

(B) Technical employees or qualified veterinarians must conduct routine clinical examinations on all test animals. Clinical examination, unless required otherwise by a specific test standard, must include weighing of each animal, at approximately the same time of day, at least once a week during the first 13 weeks, and every two weeks thereafter and observing all animals in relation to food and water consumption, morbidity, mortality and causes thereof, loss of animals for whatever reason, signs of toxicity, pharmacologic effects, and behavioral changes.

(iii) *Killing of test animals.* Animals which appear during the study as moribund, injured, or weak, and not expected to survive to the next observation, must be killed to preclude the loss of tissues from cannibalism and/or autolysis. Animals surviving to the termination of the study must also be killed. A technical employee or qualified veterinarian must obtain blood samples for hematologic determinations from each animal immediately before it is killed or as it is killed. The method used for killing must be humane and the same throughout the study. The tester must select a method of killing which will not produce interfering pathologic lesions.

(7) *Pathology procedures.* A Board-Certified or Board-Eligible pathologist as specified in § 772.100–2 (b)(1)(i)(A), must be responsible for the planning and conduct of all pathology procedures and histopathology examination, as well as for the final interpretation of all pathology data. Other doctorate pathologists, as specified in § 772.100–2 (b)(1)(i)(B), are also acceptable for conducting procedures in their disciplines of specialization, under the direct supervision of a Board-Certified or Board-Eligible pathologist as specified in § 772.100–2(b)(1)(i)(A).

Note.—Direct supervision means that the supervisor is immediately available for consultation, as necessary. This consultation may be done in person or by telephone.

(i) Gross necropsy.

(A) Qualified pathologists, as specified in § 772.100–2(b)(1)(i), must perform or personally supervise the necropsies. Other appropriately trained technical employees, as specified in § 772.100–2(b)(1)(iii)(A) may assist in the necropsy.

Note.—personal supervision means that the supervisor is immediately available for consultation at the site.

(B) Animals must be necropsied as soon as possible after death but no later than 16 hours after death. If necropsy cannot be performed immediately after the animal is killed or found dead, the animal must be immediately refrigerated (but not frozen) at temperatures low enough to minimize tissue autolysis (4–8° C). Animals found dead upon routine clinical examination must be necropsied as soon as possible to salvage usable tissues.

(C) The gross necropsy must include an initial physical examination of the external surfaces and all orifices followed by an internal examination of tissues and organs *in situ.* The examination must include the following: external and internal portions of all hollow organs; cranial cavity and external surfaces of the brain and spinal cord; nasal cavity and paranasal sinuses; neck with its associated organs and tissues; thoracic, abdominal, and pelvic cavities with their associated organs and tissues; and the muscular/skeletal carcass. The urinary bladder and lungs must be inflated with a proper fixative to allow for better gross examination and preservation.

(ii) Tissue preservation. A technical employee or qualified pathologist must immediately preserve all tissues and organs from all test animals in 10 percent buffered formalin or another recognized and accepted fixative appropriate for the specific tissue(s).

(iii) Preparation of tissue for microscopic examination. A pathologist or a technical employee, as specified in § 772.100–2(b)(1), must prepare all specimens for microscopic examination.

(A) *Tissue fixation and trimming.* The technical employee or qualified pathologist must fix tissues for the appropriate times for the fixative utilized. A pathologist must perform or directly and personally supervise tissue trimming. Routinely, tissues must be trimmed to a thickness of no more than 0.4 cm for subsequent processing. Parenchymal organs must be trimmed to allow for the largest surface areas possible for subsequent microscopic examination. Hollow organs must be trimmed to allow for a cross section mount from mucosa to serosa. Lymph nodes must be bisected through the hilus, if possible.

(B) *Slide preparation.* A technical employee or qualified pathologist must cut tissues routinely at a thickness of three to six micra (3 to 6μ), in no case exceeding 10μ. All tissues must be stained routinely with hematoxylin and eosin (H&E). EPA encourages the use of special stains appropriate to the specific neoplasm, lesion, or tissue. Multiple sections (step cuts) must be made on each tissue or organ that contains gross evidence of a neoplasm or lesion and on each tissue or organ in which a metastasis may be anticipated. The tester must identify all blocks and microscopic slides by reference to the animal's specific identification number and must preserve and hold them in accordance with § 772.110–1(j)(2).

(iv) Microscopic examination and evaluation.

(A) Qualified pathologists as described in § 772.100–2(b)(1)(i), must perform the microscopic examination and evaluation with subsequent diagnosis. The same pathologist must examine and evaluate all microscopic slides from all test animals of a given species.

(B) The pathologist must record, document, and report all microscopic findings including all abnormalities, lesions, neoplasms, metastatic tumors and their anatomic location.

(v) Additional Examinations. All adverse health effects observed during the course of the study must be examined. When there is clinical evidence of specific toxicologic or pharmacologic effects related to specific target organs, the necropsy and microscopic examinations of the suspected target organs must be

conducted in greater detail. For example, when there is clinical evidence of neurologic effects, multiple sections from brain, spinal cord, and nerves must be examined.

(8) *Reporting of data.* Each test report submitted under this subpart must satisfy the reporting requirements of this section, unless a specific test standard elsewhere in this subchapter directs otherwise.

(i) *General requirements.* (A) *Identification.* Each test must identify:

(*1*) The laboratory where the test was performed, by name and address; and

(*2*) Each party primarily responsible for any written or other matter contained in the report, and the portions of the report for which the person is responsible.

(B) *Verification.* Each test report must be: (*1*) Signed by each of the senior scientific personnel (including the laboratory director) responsible for performing and supervising the testing, and preparing, reviewing, and approving the test report; and

(*2*) Certified by the sponsor or an authorized agent of the sponsor as a complete an unaltered copy of the report provided by the testing laboratory, whether independent or owned, operated, or controlled by the sponsor.

(ii) *Format and content.* The test report must include all information necessary to provide a complete and accurate description and evaluation of the test procedures and results. A test report must contain at least three parts: A summary and evaluation of the test results; a description of the test procedures, and the data and information required by each applicable section of this subpart. Particular information, data, or analysis may be required more that it is required. Units of measurement must be in the metric system, but the English system may also be used when appropriate. In no instance must the systems be mixed (e.g., mg/sq. in.) nor should both systems be used alternately within a test report.

(A) *Summary and evaluation of test results.* This section of the test report must contain a summary and analysis of the data, and a statement of the conclusions drawn from the analysis. The summary must highlight any and all positive data or observations, and any deviations from control data which may be indicative of toxic effects. The summary must be presented in sufficient detail to permit independent evaluation of the results.

(B) *Description of the test procedure.* This section of the test report must include, but not be limited to, the following information (if a sponsor believes the reporting requirements are inapplicable, the sponsor must submit an explanatory statement to this effect):

(*1*) *Deviation from standards.* The report must indicate all ways in which the test procedure fails to meet applicable standards for acceptable testing contained in this subpart, and must state the reasons for such deviations.

(*2*) *Methodology.* Specifications of test methods, including a full description of the experimental design and procedures, the length of the study, and the dates on which the study began and ended must be stated.

(*3*) *Substance tested.* Identification of the test substance must be provided, including:

(*i*) Chemical name, chemical abstract member (CAS) or code number, molecular structure, and a qualitative and quantitative determination of its chemical composition (including names and quantities of known contaminants and impurities, so far as is technically feasible; the determinations must also include quantities of unknown materials, if any, so that 100 percent of the sample tested is accounted for);

(*ii*) Manufacturer and lot number of the substance; relevant properties of the substance tested, such as physical state, pH, stability, and purity; and

(*iii*) Identification and composition of any vehicles (e.g., diluents, suspending agents, and emulsifiers) or other materials used in administering the test substance.

(*4*) *Animal data.* Animal data must include:

(*i*) Species and strain used, rationale for selection of species (if the species is other than the species preferred or required by sections of this subpart), and rationale for selection of strain;

(*ii*) Source of supply of the animals;

(*iii*) Description of any pretest conditioning, including diet and quarantine;

(*iv*) Method of randomization used in assigning animals to test or control groups;

(*v*) Numbers of animals of each sex in each test or control group; and

(*vi*) Age and condition of animals at beginning of study.

(*5*) *Environmental conditions.* A description of the caging conditions must include: Number (and any change in number) of animals per cage, bedding material, ambient temperature, and humidity, photoperiod, and identification and description of the diet of the test animal.

(*6*) *Dosing.* Dosing information must include:

(*i*) All dose levels administered;

(*ii*) Method and frequency of administration (including hour of dosing in relation to photoperiod);

(*iii*) Total volume of material (i.e., test substance plus vehicle) contained in individual dosings;

(*iv*) Duration of treatment;

(*v*) If the test substance is administered in the feed or by another vehicle, the method of randomization used in selecting samples to assay, the assay method used to determine the stability and homogeneity of the test substance being administered, and the results of this assay;

(*vi*) For each dose level, the mean total amount of test substance administered per animal; and

(*vii*) The rationale (including discussion of alternatives) for selection of the vehicle.

(*7*) *Treatment for infectious diseases.* A description of the treatment(s) used to prevent or control infectious diseases if such treatment was undertaken during a test or shortly before a test was begun. Such a description must include, for each individual affected animal:

(*i*) Its identification number;

(*ii*) The nature and severity of the disease, if present;

(*iii*) The date of first observation and duration of disease, if present;

(*iv*) The nature of the treatment for disease or disease prevention, and the dates of such treatment; and

(*v*) The outcome of the treatments in relation to the disease and the test results.

(*8*) *Observations.* Frequency, duration, and method of observation of the animal.

(*9*) *References.* Statistical and any other methods employed for analyzing the raw data; a list of references to any published literature used in developing the test protocol, performing the testing, making and interpreting observations, and compiling and evaluating the results.

(C) *Reporting requirements for specific tests.* This section of the test report must include all data, information, and analysis required by the "Data reporting and evaluation" paragraphs of the sections in this subchapter.

(iii) *Statistical procedures.* (A) *General.* Statistical techniques are required for several toxicological analyses, such as the LD_{50} calculations for acute oral and acute dermal toxicity studies (§§ 772.112–21 and 772.112–22), the LC_{50} calculations for acute inhalation toxicity study (§ 772.112–23), and the median particle size analyses used to describe the aerosol clouds in the acute and subchronic inhalation

studies (§§ 772.112–23 and 772.112–33). Median lethal doses are to be measured within a 95 percent confidence limit of 20 percent of the median. When not feasible, e.g., due to inherently variable responses or to difficulties in administering the test substance, the tester must explain why the limit was exceeded. In addition, appropriate statistical methods must be used to summarize experimental data, to express trends, and to evaluate the significance or differences in data from individual test groups. The methods used must reflect the current state of the art. A list of references in the appendix to this section represents some of the techniques currently in use.

(B) *Standard deviation and standard error.* All data averages or means must be accompanied by standard deviations, to indicate the amount of variability in the raw data. In addition, the standard errors of the means should also be calculated, since they are useful in comparing means from different test groups; however, notations of statistically significant differences, accompanied by the confidence level or probability, may be used in place of the standard errors. Other methods of expressing data dispersion may also be used, when appropriate.

Appendix A

(1) The following are a few of many good textbooks in statistics:

(i) Remington, R. D., and M. A. Schork. 1970. Statistics with Applications to the Biological and Health Sciences. Prentice-Hall, New York. (Includes a chapter on nonparametric methods [Chapter 12. Distribution Free and Nonparametric Methods] which are useful for nonnormally distributed data.)

(ii) Rohlf, F. J. and R. R. Sokal. 1969. Statistical Tables. W. H. Freeman & Co., San Francisco.

(iii) Sokal, R. R. and F. J. Rohlf. 1969. Biometry. W. H. Freeman & Co., San Francisco.

(iv) Von Fraunhofer, J. A., and J. J. Murray. 1976. Statistics in Medical, Dental, and Biological Studies. Tri-Med Books Limited, London.

(2) The following are examples of available computer programs which can be used in the statistical processing of data but generally involve a large computer operation. There are also many desk-top minicomputers which supply similar computer programs for statistical analyses.

(i) Dixon, W. J., ed. 1970. Biomedical Computer Programs (BMD). 2nd Ed. University of California Press, Los Angeles.

(ii) Nie, N. H., C. H. Hull, J. G. Jenkins, K. Steinbrenner, and D. H. Bent. 1975. Statistical Package for the Social Sciences (SPSS). 2nd Ed. McGraw-Hill, New York.

Appendix B

Dietary Requirements and Contaminant Analysis

Dietary Requirements. This Appendix provides dietary requirements and contaminant analysis of feeds and vehicles for use as required in specific test standards.

(a) *Nutrient Requirements.* The diet used must be manufactured from the following ingredients and no others. (Reference: Nutrient Requirements of Laboratory Animals, Third Revised Edition, National Academy of Sciences, Washington, D.C., 1978.)

(1) *Ingredients:*

Fish meal,
Soybean meal,
Alfalfa meal,
Corn gluten meal,
Ground whole wheat,
Ground No. 2 yellow shelled corn,
Ground whole oats,
Wheat middlings,
Brewers dried yeast,
Soy oil,
Salt, Dicalcium phosphate, and
Ground limestone.

(i) A vitamin premix must be prepared from the following vitamin sources:

Vitamin A Palmitate or Acetate,
D activated animal sterol,
Menadione activity,
Alpha-tocopherol acetate,
Choline Chloride,
Folic Acid,
Niacin,
d-Calcium Pantothenate,
Riboflavin Supplement,
Thiamine mono nitrate,
B12 Supplement,
Pyridoxine hydrochloride, and
d-Biotin.

A mineral premix must be prepared from the following compounds:

Cobalt carbonate,
Copper sulfate,
Iron sulfate,
Magnesium oxide,
Manganese oxide,
Zinc oxide, and
Calcium iodate.

Microanalysis—The total calculated concentration of nutrients in the diet are from the ingredients and from the vitamin and mineral fortifications at the time of manufacture must be as follows:

	Minimum (percent)
Crude protein	18.0
Crude fat	4.3
Crude fiber	4.2
Ash	8.0

Nutrient concentrations in final diet must not vary from the individually stated values by more than 10%.

	Minimum
Amino Acids (total diet): Arginine	0.95 percent
Lysine	.90 percent
Methionine	.38 percent
Cystine	.27 percent
Tryptophan	.20 percent
Glycine	1.00 percent
Histidine	.38 percent
Leucine	1.50 percent
Isoleucine	.95 percent
Phenylalanine	.90 percent
Tyrosine	.60 percent
Threonine	.65 percent
Valine	.95 percent
Minerals:	
Calcium	1.15 percent
Phosphorus	.90 percent
Potassium	.80 percent
Sodium	.33 percent
Magnesium	.20 percent
Iron	345 mg/kg
Zinc	50 mg/kg
Manganese	140 mg/kg
Copper	12 mg/kg
Cobalt	.80 mg/kg
Iodine	1.85 mg/kg

	Minimum	Maximum
Vitamins:		
Vitamin A	15.0	75.00 IU/g
Vitamin D	4.0	10.00 IU/g
Alpha-tocopherol	50.0	mg/kg
Thiamine	14.0	mg/kg
Riboflavin	7.0	mg/kg
Niacin	65.0	mg/kg
Pantothenic Acid	32.0	mg/kg
Choline	1900.0	mg/kg
Pyridoxine	10.0	mg/kg
Folic Acid	2.0	mg/kg
Biotin	0.3	mg/kg
Vitamin B12	14.0	Mcg/lb
Vitamin K	3.0	mg/kg
For autoclavable diet:		
Vitamin A	30.0	mg/kg
Thiamin	70.0	mg/kg
Vitamin K	20.0	mg/kg

The diet must be void of any feed additives containing antibiotics or estrogen activity. When diet is purchased for feeding in meal form, it must be manufactured by regrinding pellets.

(2) *Approximate Analysis*—Analysis for nutrient content of both ingredients and the finished product must be conducted in accordance with the procedures of the Association of Official Agricultural Chemists (1975) and must be expressed as a nutrient content percentage by weight on an air-dry basis.

(3) *Ingredient Standards*—Ingredients used in the manufacture of this ration must not be contaminated with any more than 3 percent of foreign materials such as other grains, weed seeds, chaff, etc. Nor will any mold, must, or insect/rodent infestation be allowed. The average minimum nutrient concentrations of ingredients used in the manufacture of this ration must be equal to the values published in the National Academy of Sciences Publication 1684, United States-Canadian Tables of Food Composition.

The data from these analyses must be made available to the facility using the product.

(4) *Feed Additives and Processing Restrictions*—The product must contain no antibiotics, other preservatives or estrogen additives of any kind. All milling and warehousing conditions and/or restrictions, as specified in the latest issue of the national Institutes of Health Standard No. 1, apply to the feed covered by this specification. The product must not be altered in any manner that will affect the final nutrient content.

(5) *Labeling*—Each bag must be clearly marked with the name of the product, the name of the manufacturer, the net weight, the ingredients, the guaranteed analysis of its contents, the date (month, day, and year) the manufacturing process was completed, and the batch number under which it was processed.

(b) *Contaminant analysis of feeds and vehicles.*

Parameter	Specification limitation	
	Minimum	Maximum
Aflatoxin (B1, B2, G1, G2) ppb Total		5
Estrogenic Activity, ppb (DES eq.)		1
Lindane, ppb		20
Heptachlor, ppb		20
Malathion, ppm		2.5
DDT (Total), ppb		100
Dieldrin, ppb		20
Cadmium, ppb		160
Arsenic, ppm		1.0
Lead, ppm		1.5
Mercury, ppb		100
Selenium, ppm	0.1	0.6
PCB, ppb		50
Nitrosamines, ppb		10

Subpart C—Acute and Subchronic Health Effects

§ 772.112–10 General.

(a) *Scope and purpose.* The standards in this subpart are designed to develop data on acute and subchronic health effects of chemical substances and mixtures ("chemicals") subject to acute and subchronic health effects test regulations under the Toxic Substances Control Act (TSCA) (P.L. 94–469, 90 Stat. 2003, 15 U.S.C. 2601 et. seq.). The EPA will use these data to assess the risk of acute and subchronic effects these chemicals may present to human health.)

(b) *Applicability.* These standards apply to the development of acute and subchronic health effects data from the testing of chemicals specified in Part 771 of this subchapter.

(c) *Definitions.* The definitions in Section 3 of the TSCA, and the definitions in § 770.2, § 772.113–1(c), and § 772.110–1(a)(3), entitled "Good Laboratory Practice Standard for Health Effects," apply to Subpart C. In addition, the following definitions also apply to Subpart C:

(1) "Acute effects" means short-term health effects following a single administration of a test substance;

(2) "Acute toxicity" means the total of adverse effects following a single administration of a test substance;

(3) "Subchronic effects" means health effects following continuous or repeated administration of a test substance over a period of approximately 90 days;

(4) "Subchronic toxicity" means the total of adverse effects following continuous or repeated administration of a test substance over a period of approximately 90 days;

(d) *Good Laboratory Practice Standards for Acute and Subchronic Health Effects.* The Good Laboratory Practice Standards for Health Effects in § 772.110–1, Subpart B, apply to Subpart C.

(e) *Specific Personnel Requirements.* The specific personnel requirements in Subpart A, § 772.110–2(b), apply to Subpart C.

(f) *Test or Control Substance Concentration.* Section 772.100–2(b)(3) of Subpart A apply to Subpart C.

§ 772.112–21 Acute oral toxicity study.

(a) *Study design.* (1) *Species.* Testing must be performed with the laboratory rat.

(2) *Sex and age.* Young adult male and female animals must be used.

(3) *Number of animals and selection of dose levels.* (i) A trial test is recommended for the purpose of establishing a dosing regimen which must include one dose level higher than the expected LD_{50}. If data based on testing with at least 5 animals per sex are submitted showing that no toxicity is evident at 5g/kg, no further testing at other dose levels is necessary. If mortality is produced, the requirements of paragraph (a)(3)(ii) of this section must apply.

(ii) Enough animals per dose level and sufficient dose levels spaced appropriately must be used to produce test groups with mortality rates between 10 percent and 90 percent and to permit the calculation of the LD_{50} for males and females with a 95 percent confidence interval of 20 percent or less. At least 3 dose levels of the test substance, in addition to controls (if any), must be tested. Though the group sizes may vary for each dose level, each group must contain equal numbers of male and female animals.

(4) *Control animals.* (i) A concurrent vehicle control group is recommended if the vehicle or diluent used in administering the test substance would be expected to elicit any important acute toxicologic response, or if there are insufficient data on the acute effects of the vehicle.

(ii) A concurrent untreated control group is not required.

(5) *Dosing.* All animals must be dosed by gavage. All animals must receive the same concentration of dosing solution. They should also receive about the same volume of dosing solution, which should not exceed 4–5 ml per animal.

(6) *Duration of test.* The animals must be observed for at least 14 days after dosing, or until all signs of reversible toxicity subside, whichever occurs later.

(b) *Study Conduct.* (1) *Fasting.* Food shall be withheld from the animals the night prior to dosing.

(2) *Observation.* The animals must be observed frequently during the day of dosing and checked at least every 12 hours throughout the test period. The following must be recorded: Nature, onset, severity, and duration of all gross or visible toxic or pharmacological effects, e.g., abnormal or unusual cardiovascular, respiratory, excretory, behavioral, or other activity, as well as signs indicating an adverse effect on the central nervous system (paralysis, lack of coordination, staggering); pupillary reaction; and time of death. The weight of each animal must be determined at least semi-weekly (3–4 day intervals) throughout the test period, and at death.

(3) *Sacrifice and necropsy.* All test animals living at the termination of the observation period must be sacrificed. All test animals, whether dying by sacrifice or during the test must be subjected to a complete gross necropsy following their death, in accordance with § 772.100–2(b)(7), Subpart A. All abnormalities must be recorded.

(c) *Data reporting and evaluation.* In addition to the information required by § 772.100–2(b)(8), Subpart A, the test report must include the following information:

(1) Tabulation of response data by sex and dose level (i.e., number of animals dying per number of animals showing signs of toxicity per number of animals exposed);

(2) Time of death after dosing.

(3) LD_{50} for each sex for each test substance calculated at the end of the observation period (with method of calculation specified);

(4) 95 percent confidence interval for the LD_{50}; and

(5) Dose-response curve and slope.

Appendix

(1) The following publications provide information on acceptable methods of calculating the LD_{50}:

(i) Finney, D.J. 1971. Probit Analysis. 3d Ed., Chapters 3 and 4. Cambridge University Press: Cambridge, Eng.

(ii) J. T. Litchfield, Jr., and F. Wilcoxon. 1949. A simplified method of evaluating dose-effect experiments. J. Pharmacol. Exp. Therap. 96:99–115.

(iii) Thompson, W. R. 1947. Use of moving averages and interpolation to estimate median effective dose. Bacteriological Rev. 11:115–145.

(iv) Weil, C.S. 1952. Tables for convenient calculation of median effective dose and instructions in their use. Biometrics 8:249–263.

(2) The following publications contain additional useful information:

(i) Boyd, E. M. 1972. Predictive Toxicometrics (Chapters 14, 15, and 16). Bristrol, Scientechnia Publisher, Ltd. (dist.: Williams and Wilkens Co., Baltimore.)

(ii) Committee for the Revision of NAS Publication 1138, Committee on Tox., Nat. Res. Council. 1977. Principles and Procedures for Evaluating and Toxicity of Household Substances. Pp. 1–18. Prepared for the Consumer Product Safety Commission. Nat. Acad. Sci.: Washington, D.C.

(iii) Hagan, E. C. 1965. Appraisal of the Safety of Chemicals in Foods, Drugs, and Cosmetics. pp. 17–25. Association of Food and Drug Officials of the United States. Topeka, Kans.

(iv) Loomis, T. A. 1974. Essentials of Toxicology. 2nd Ed. Pp. 145–215. Lea & Febinger: Philadelphia.

(v) Paget, G. E. ed., 1970. Methods in Toxicology. Pp. 49–82. F. A. Davis & Co.: Philadelphia.

(vi) Weil, C. S. 1972. Guidelines for experiments to predict the degree of safety of a material for man. Toxicol. Appl. Pharmacol. 21:194–199.

(vii) Weil, C. S. and G. J. Wright. 1967. Intra- and interlaboratory comparative evaluation of a single oral test. Toxicol. Appl. Pharmacol. 11:378–388.

§ 772.112–22 Acute dermal toxicity study.

(a) *Study design.* (1) *Condition of test substance.* If the test substance is a liquid, it must be applied as a liquid. If the test substance is a solid, it must be slightly moistened (made pasty) with physiological saline before application.

(2) *Species.* Testing must be performed with at least one mammalian species, preferably albino rabbits. An alternative species may be used if the sponsor can provide sufficient data and/or rationale to demonstrate that it is a more appropriate species for a specific test substance.

(3) *Age.* Young adult male and female animals must be used.

(4) *Number of animals and selection of dose levels.* (i) A trial test is recommended for the purpose of establishing a dosing regimen which must include one dose level higher than the expected LD_{50} and at least one dose level below the expected LD_{50}. If data based on testing with at least 5 animals per sex with abraded skin are submitted showing that no toxicity is evident at 2 g/kg, no further testing at other dose levels is necessary. If mortality is produced, the requirements of paragraph (a)(4)(ii) of this section apply.

(ii) The number of animals per dose level, and the number and spacing of dose levels must be chosen to produce test groups with mortality rates between 10 percent and 90 percent, and permit calculation of the LD_{50} (abraded skin and intact skin) of males and females with a 95 percent confidence interval of 20 percent or less. At least 3 dose levels of the test substance, in addition to controls, must be tested. Though the group sizes may vary for each dose level, the groups must contain equal numbers of male and female animals.

(5) *Control animals.* A concurrent untreated control group of animals is required. A concurrent vehicle control group is required if a vehicle or diluent used in administering the test substance would be expected to elicit any important acute toxicologic response, or if there are insufficient data on the acute effects of the vehicle.

(b) *Study conduct.* (1) *Application.* In all animals, the application site must be free of hair. In addition, the application sites in abraded-skin groups must be abraded in such a way as to penetrate the stratum corneum but not the dermis. The test substance must be kept in contact with the skin of at least 10 percent of the body surface (for rabbits) for at least 24 hours. (See Draize (1944) for equivalent sq. cm of body surface.) The preferred application site is a band around the trunk of the test animal. A wrapping material such as gauze covered by impervious, nonreactive rubberized or plastic material should be used to retard evaporation and keep the test substance in contact with the skin. At the end of the exposure period, the wrapping should be removed and the skin wiped (but not washed) to remove any test substance still remaining.

(2) *Duration of observation.* Animals must be observed for at least 14 days after dosing or until all signs of reversible toxicity in survivors subside, whichever occurs later.

(3) *Observation.* Animals must be observed frequently during the day of administration of the test and checked at least every 12 hours throughout the test period. The following must be recorded: Nature, onset, severity, and duration of each toxic and pharmacologic sign, such as abnormal or unusual cardiovascular, respiratory, excretory, behavioral, or other activity, as well as signs indicating an adverse effect on the central nervous system (paralysis, lack of coordination, staggering); pupillary reaction; and time of death. The weight of each animal must be determined at least semi-weekly (3–4 day intervals) throughout the test period, and at death.

(4) *Sacrifice and necropsy.* All animals living at the termination of the observation period must be sacrificed. All test animals, whether dying by sacrifice or during the test, must be subjected to a complete gross necropsy following their death, in accordance with § 772.100–2(b)(7), Subpart A. All abnormalities must be recorded.

(5) *Histopathology.* Examination of skin must include histological examination of treated tissue in accordance with § 772.100–2(b)(7), Subpart A.

(c) *Data reporting and evaluation.* In addition to the information required by § 772.100–2(b)(8) and paragraphs (b) (3), (4), and (5) of this section, the test report must include the following information.

(1) Tablulation of response data by sex and dose level (i.e., number of animals dying per number of animals showing signs of toxicity per number of animals exposed);

(2) Time of death after treatment;

(3) Time of recovery for fully recovered animals;

(4) LD_{50} for each sex for each test substance for animals with abraded skin and for animals with intact skin, calculated at the end of the observation period (with method of calculation specified);

(5) 95 percent confidence interval for each LD_{50}; and

(6) Dose-response curve, and slope (with confidence limits).

Appendix

(1) Committee for the Revision of NAS Publication 1138. Committee on Tox. Nat. Res. Council. 1977. Principles and Procedures for Evaluating the Toxicity of Household Substances. Pp. 1–9, 23–55. Prepared for the Consumer Product Safety Commission Nat. Acad. Sci., Washington, D.C.

(2) Draize, J. H., G. Woodard, and H. O. Calvery. 1944. Methods for study of irritation and toxicity of substance applied topically to skin and mucous membranes. J. Pharmacol. Exp. Ther. 83:377–390.

(3) Draize, J. H. 1965. Appraisal of the Safety of Chemicals in Foods, Drugs, and Cosmetics—Dermal Toxicity. Pp. 46–59. Assoc. of Food and Drug Officials of the United States. Topeka, Kans.

(4) Marzulli, F. N., and H. I. Maibach. 1977. Dermato-Toxicology and Pharmacology (Advances in Modern Toxicology). Vol. 4. Hemisphere Publishing Corp., Washington, D.C.

§ 772.112–23 Acute inhalation toxicity study.

(a) *Study design.* (1) *Species, sex, and age.* Testing must be performed with the laboratory rat. Young adult male and female animals must be used.

(2) *Number of animals and selection of dose levels.* (i) A trial test is recommended for the purpose of establishing a dosing regimen which must include one dose level higher than the expected LC_{50} and at least one dose level below the expected LC_{50}. If data based on testing with at least 5 animals per sex are submitted showing that no

toxicity is evident at 5 mg/1, no further testing at other dose levels is necessary. If mortality is produced, the requirements of paragraph (a)(2)(ii) of this section apply.

(ii) The number of animals per dose level, and the number and the spacing of dose levles must be chosen to produce test groups with mortality rates between 10 percent and 90 percent, and to permit calculation of the LC_{50} with a 95 percent confidence limit of 20 percent or less. At least 4 dose levels of the test substance, in addition to controls, must be tested. Though the group sizes may vary for each dose level, the group must contain an equal number of male and female animals.

(3) *Duration of test.* In selecting the exposure period, allowance must be made for changed concentration equilibration time. Where there is no difficulty in maintaining a steady concentration of the test substance in the chamber(s), the exposure period must be at least 1 hour. Where there is some difficulty in maintaining a study concentration the exposure period must last up to 4 hours. The animals must be observed for 14 days, or until all signs of reversible toxicity subside, whichever occurs later.

(4) *Use of solvent.* A solvent may be added to the test substance, if necessary, to help generate an exposure atmosphere. If a product's labeling instructions specify the use of a particular solvent, that solvent is preferred. If no solvent is specified in the product's labeling instructions, the solvent, if any, which is used to formulate the product should be used.

(5) *Control groups.* (i) A concurrent untreated control group is required.

(ii) If any solvent, other than water, is used in generating the exposure atmosphere, a vehicle control group must be tested. The vehicle control group must be exposed to an atmosphere containing the greatest concentration of solvent present in any test system.

(b) *Study conduct.* (1) *Exposure chamber design and operation.*

(i) Inhalation exposure techniques described in this section are based on the use of whole-body inhalation chambers which allow the experimental animals to receive whole-body dermal exposure and possible large oral exposure, as well as the exposure by inhalation. In some cases, the investigators will want to use other inhalation exposure techniques involving face masks, head-only exposure, intratracheal instillation, or other similar techniques which reduce or preclude added dermal and oral exposures. Some alternative techniques are described by Phalen, 1976. When alternative techniques are used, the procedures and results must be reported in a manner similar to that required with the use of whole-body inhalation chambers.

(ii) Animals must be tested in a dynamic air flow exposure chamber. The chamber design must be chosen to enable production of an evenly distributed exposure atmosphere throughout the chamber. The chamber design also should minimize crowding of the test animals and maximize their exposure to the test substance.

(2) *Operation measurements.* The following measurements must be taken with care to avoid major fluctuations in the air concentrations or major discrepancies in the operation of the chambers.

(i) *Air flow.* The rate of air flow through the chamber must be measured continuously.

(ii) *Chamber concentrations.* (A) Nominal concentrations must be calculated for each run by dividing the amount of the test substance used for the generating system by the air flowing through the chamber during the exposure.

(B) Actual chamber concentrations must be determined by samples of chamber air taken near to the breathing zone of the animals as frequently as necessary to obtain an averaged integrated external exposure which is representative of the entire exposure period. The system used to generate the vapor, gas, or aerosol should be such that the chamber concentrations and particle size distributions are controlled under stable conditions, reflecting the current state-of-the-art, and should not vary in a range greater than 30 percent of the average (range/mean equal to or less than 30 percent).

(iii) *Temperature and Humidity.* The temperature must be maintained at 24 ± 2° C, and the humidity within the chamber at 40–60 percent. Both must be monitored continuously.

(iv) *Oxygen.* The rate of air flow through the chamber must be adjusted to insure that the oxygen content of exposure atmosphere is at least 19 percent.

(v) *Particle Size Measurement.* (A) *General.* In the case of gases and vapors, particulate sampling should be carried out at intervals to insure the animals are not being exposed to unknown and unexpected particulate materials. Aerosol particle size measurements should be made on samples taken at the breathing level of the animals. These analyses should be carried out using techniques and equipment reflective of the state-of-the-art. All of the suspended aerosol (on a gravimetric basis) should be accounted for, even when most of the aerosol is not respirable.

(B) *Sizing Analysis.* The sizing analysis should be in terms of equivalent aerodynamic diameters and should be represented as geometric mean (median) diameters and their geometric standard deviations (see NIOSH syllabus in the Appendix to this section), as calculated from log probability graphs or computer programs. The size analyses should be carried out frequently during the development of the generating system to insure proper stability of aerosol particles, and only as often thereafter during the exposure as is necessary to determine adequately the consistency of particle distributions to which the animals are exposed, maintaining at least 20 percent of the particles at 10 microns or less. At a minimum, these analyses should be carried out once per hour for each level of exposure for gasious test substances, twice per hour for liquid test substances, and 4 times per hour for dusts and powders.

(3) *Observation.* The animals must be observed frequently during the day of dosing and checked at least every 12 hours throughout the test period, for at least 14 days after dosing or until all signs of reversible toxicity subside, whichever occurs later. The following must be recorded: Nature, onset, severity, and duration of all gross or visible toxic or pharmacologic effects, i.e., abnormal or unusal cardiovascular, respiratory, excretory, behavioral, or other activity, as well as signs indicating an adverse effect on the central nervous system (paralysis, lack of coordination, staggering); pupillary reactions; and time of death. The weight of each animal must be determined on the day of dosing, 2, 3, 4, 7, and 14 days after dosing, weekly thereafter, and at death.

(4) *Sacrifice and Necropsy.* All animals living at the termination of the observation period must be sacrificed. All test animals, whether dying by sacrifice or during the test, must be subjected to a complete gross necropsy following their death, in accordance with § 772.100–2(b)(7), Subpart A. Examination must include nasal passages, trachea, bronchi, and lungs, and any other tissues known to be affected by the test substance. All abnormalities must be recorded.

(5) *Preservation of tissues and histopathology examination.* The following are required:

(i) Those tissues designated in paragraph (b)(5)(ii) of this section must be placed in suitable fixative as soon as possible. Tissues and microscopic slides must be prepared according to the standards set forth in § 772.100–2(b)(7)(ii) and (iii), Subpart A. Tissue samples, tissue blocks, and microscopic slides must be preserved and held in accordance with § 772.110–1(j).

(ii) The following tissues must be examined microscopically:

(A) Lungs, liver, and kidneys at all dose levels.

(B) Any tissue or organ that appears abnormal, at any dosage level, as determined in the necropsy examination.

(iii) The histopathology findings must be recorded and reported as required by paragraph (c)(10) of this section.

(c) *Data reporting evaluation.* In addition to information required by § 772.100–2(b)(8), Subpart A, and paragraphs (b)(3) and (b)(4) of this section, the test report must include the following:

(1) Vapor pressure and particulate size (median size with geometric standard deviation).

(2) Description of the chamber design and operation, including type of chamber, its dimensions, the source of makeup air and its conditioning (heating or cooling) for use in the chamber, the treatment of exhausted air, the housing and maintenance of the animals in the chambers, and similar related information. Equipment for measuring temperatures and humidity, the generating system, and the methods of analyzing airborne concentrations and particle sizing must be described;

(3) The following operation data must be tabulated both individually and in summary form, using means and standard deviations (with or without ranges) in tabular form. The data summaries must be grouped according to experimental groups, and nonexpected differences (such as in temperature and airflow) and must be tested for statistical significance.

(i) Airflow rates through the chamber;

(ii) Chamber temperature and humidity;

(iii) Nominal concentrations;

(iv) Actual concentrations; and

(v) Median particle sizes and their geometric standard deviations and percent of particles 10 microns or less.

(4) Tabulation of the response data (number of animals dying per number of animals showing signs of toxicity per number of animals exposed) at each exposure level by sex, and time of death after dosing;

(5) Tabulation of the body weights on the day of dosing, 2, 3, 4, 7, and 14 days after dosing, weekly thereafter, and at death.

(6) The LC_{50} (calculated on an exposure of one hour) for each sex for each test substance;

(7) Specification of the method used for LC_{50} calculation;

(8) The 95 percent confidence interval for the LC_{50};

(9) The dose-response curve and slope (with confidence limits); and

(10) The histopathology findings including a complete record of lesions and abnormalities observed, and the histological diagnosis and characterization of each kind of lesion or abnormality observed, naming those which apparently caused death or morbidity.

Appendix

The following texts and articles give the reader some breadth of scope as well as sufficient detail to develop and carry out inhalation toxicity studies.

(1) General references

(i) Altman, P. L., *et al.* 1958. Handbook of Respiration. Aero Medical Laboratory: Wright-Patterson Air Force Base, Ohio. (Includes extensive data on humans and animals).

(ii) Casarett, L. J. and J. Doull. 1975. Toxicology. Basic Science of Poisons. MacMillan Publishing Co., Inc.: New York. (Good text on general toxicology, includes Chapter 9, "Toxicology of the Respiratory System.")

(iii) Committee for the Revision of NAS Publication 1138, Committee on Tox., Nat. Res. Council. 1977. Principles and Procedures for Evaluating the Toxicity of Household Substances. Pp. 8–22, 61–75. Prepared for the Consumer Product Safety Comm. Nat. Acad. Sci.: Wahington, D.C.

(iv) Comroe, J. H. 1974. Physiology and Respiration. 2d Ed. Year Book Medical Pub.: Chicago. (Includes the anatomy, physiology, physiological testing, and pathology of the lungs in humans.)

(v) Hatch, T. G., and P. Cross, 1964. Pulmonary Deposition and Retention of Inhaled Aerosol. Academic Press: New York. (Comprehensive text which deals with the anatomy, physiology, deposition, and retention and pathological changes in the lung.)

(vi) Hayes, W. J. 1975. Toxicology of Pesticides. Williams and Wilkins Company: Baltimore. (This is good general text for pesticide toxicity and includes sections on inhalation toxicity.)

(vii) ICRP Committee, P. Morrow (Chairman). 1966 and 1967. Deposition and retention models for internal dosimetry of the human respiratory tract. Health Phys. 12:173–207 (1966). Errata and revisions to report, Health Phys. 13:1251. (1967)

(viii) MacFarland, H. N. 1976. Chapter 5: Respiratory Toxicology. Pp. 121–154 in Essays in Toxicology, Vol. 7. Hayes, W. J., ed. Academic Press: New York.

(ix) National Institute of Occupational Health and Safety. 1973. The Industrial Environment—Its Evaluation and Control. Supt. Doc., Gov. Print. Off.: Washington. D.C. (Contains many practical chapters which cover the evaluation of the environment and the effects of chemicals.)

(2) Advanced monographs.

(i) Davies, C. N., ed. 1961. Inhaled Particles and Vapours. Proceedings of an International Symposium organized by the British Occupational Hygiene Society, Oxford, March 29–April 1, 1960. Pergamon Press: New York.

(ii) Davies, C. N., ed. 1967. Inhaled Particles and Vapours II. Proceedings of an International Symposium organized by the British Occupational Hygiene Society, Cambridge, September 28–October 1, 1965. Pergamon Press: New York.

(iii) Hanna, M. G., P. Nettesheim, and J. R. Gilbert, Jr. 1970. Inhalation Carcinogenesis. USAEC, Conf. No. 691001. Clearinghouse for Federal Scientific and Technical Information: Springfield, VA.

(iv) Hook, G. E. R., and G. W. Lucier, eds. 1976. Target Organ Toxicity: Lung. Environ. Health Perspec. 16:1–187.

(v) Mercer, T. T., P. E. Morrow, and W. Stober. 1972. Assessment of Airborne Particles. Charles C. Thomas: Springfield, Ill.

(vi) Walton, W. H., ed. 1970. Inhaled Particles III. Proceedings of an International Symposium organized by the British Occupational Hygiene Society , London, September 14–23, 1970. Volumes I and II. Unwin Brothers Limited, Gresham Press: Surrey, England.

(vii) Walton, W. H., ed. 1975. Inhaled Particles IV. Proceedings of an International Symposium organized by the British Occupational Hygiene Society, London, September 22–26, 1975. Volumes I and II. Pergamon Press: New York.

(3) Exposure systems.

(i) Drew, R. T., and S. Laskin. 1973. Environmental inhalation chambers. Pp. 1–41 in Methods of Animal Experimentation, Vol. IV. Academic Press Inc.: New York and London.

(ii) Fraser, D. A., R. E. Bales, M. Lippmann, and H. E. Stokinger. 1959. Exposure Chambers for Research in Animal Inhalation. Public Health Service Monograph No. 57. U.S. Government Printing Office: Washington, D.C.

(iii) Gage, J .C. 1970. Experimental Inhalation Toxicity. Pp. 258–277 in Methods in Toxicology. G. E. Paget, ed. Blackwell Sci. Pub.: Oxford and Edinberg.

(iv) Hinners, R. G., J. K. Burkart, and C. L. Punte. 1968. Animal inhalation exposure chambers. Arch. Environ. Health 16:194–206.

(v) Phalen, R. F. 1976. Inhalation exposure of animals. Environ. Health Perspect. 16:17–24.

(vi) Roe, F. J. C. 1958. Inhalation tests. In Modern Trends in Toxicology, Vol. 1. Boyland, E. and R. Goulding, eds. Appleton-Century-Crofts: New York.

(4) Generating systems. Gas and vapor generating is relatively simple compared to the large number of different systems needed to generate aerosols of solids and liquids. Besides the following references, all of the

advanced monographs in paragraph (3) contain descriptions of generating systems.

(i) Drew, R. T., and M. Lippmann. 1972. Section I Calibration of air sampling instruments. II. Production of test atmospheres for instrument calibration. In: Air Sampling Instruments, 4th Ed. ACGIH 1972.

(ii) Fraser, D. A., *et al.* 1959. Exposure Chambers for Research in Animal Inhalation. Public Health Monograph No. 57. Supt. Doc., U.S. Gov. Print. Off.: Washington, D.C.

(iii) Gage, J. C. 1970. Experimental inhalation toxicity. Pp 258–277 in Methods in Toxicology. G. E. Paget, Ed. Blackwell Sci. Pub.: Oxford and Edinburg.

(iv) Raabe, O. G. 1970. Generation and characterization of aerosols. Pp. 123 in Inhalation Carcinogenesis, USAEC Conf. 691001. M. G. Hanna *et al.* Clearinghouse for Federal Scientific and Technical Information: Springfield, Va.

(5) Sampling methods.

(i) Lippman, M. 1972. Respiratory dust sampling.

Section G in Air Sampling Instruments, 4th ed. American Conference of Governmental Hygienists: Cincinnati.

(ii) Morrow, P. E. 1964. Evaluation of inhalation hazard based upon the respirable dust concept and the philosophy and application of selective sampling. Amer. Ind. Hyg. Assoc. 25:213–236.

(iii) National Institute of Occupational Health and Safety. 1973. The Industrial Environment—Its Evaluation and Control. Supt. Doc., Gov. Print. Off. (Has several good chapters on sampling, and on chemical, instrumental, and physical analyses of atmospheres.)

(iv) Peterson, C. M. 1972. Aerosol sampling for particle size analyses. Section F. Respiratory dust sampling. In Air Sampling Instruments, 4th ed. American Conference of Governmental Hygienists: Cincinnati.

(v) Preining, O., D. Sheesley, N. Djordevic, *et al.* 1967. The size distribution of aerosols produced by air blast nebulization. J. Colloid and Interface Sci. 23:3.

(vi) Silverman, L., C. E. Billings, M. W. First, *et al.* 1971. Particle Size Analysis in Industrial Hygiene. Academic Press, Inc.: New York and London. (Sampling size analysis and instrumentation with an emphasis on hygiene and air cleaning.)

(6) Pulmonary function testing. Pulmonary function tests have been widely used in the evaluation of human respiratory function but less widely used in animal research. Such analyses may be required to indicate subtle damage to the pulmonary system.

(i) Alarie, Y., A. Krumm, H. Jennings, R. Haddock, *et al.* 1971. Distribution of ventilation in cyanomologus monkeys. Arch. Environ. Health 22:633. (Illustrates testing in a primate.)

(ii) Amdur, M. O., and J. Mead. 1958. Mechanics of respiration in unanesthetized guinea pigs. Am. J. Physio. 192:364. (Illustrates function testing in a rodent.)

(iii) Comroe, J. H., *et al.* 1962. The Lung, Clinical Physiology and Pulmonary Function Tests. 2d Ed. Year Book Med. Publ., Inc.: Chicago. (Provides a discussion of pulmonary testing along with anatomy and physiology of the human respiratory system.)

(iv) Comroe, J. H. 1965. Physiology of Respiration. Year Book Med. Publ., Inc.: Chicago. (Similar to Comroe *et al.*, 1962 (above).)

(v) Mauderly, J., and J. Pickrell. 1973. Pulmonary function testing of unanaesthetized beagle dogs. In: Research Animals in Medicine. L. Harmison, ed. DHEW Pub. No. NIH 72–333. (Illustrates function testing in the dog.)

§ 772.112–24 Primary eye irritation study.

(a) *Study Design.* (1) *Condition of test substance.*

(i) If the test substance is a liquid, it must be placed in the eye undiluted, in accordance with paragraph (b) of this section.

(ii) If the test substance is a solid or granular product, it must be ground into a fine dust or powder. The test substance must not be moistened before it is placed in the eye in accordance with paragraph (b) of this section.

(2) *Species.* Testing must be performed with the albino rabbit.

(3) *Age and condition of animals.* Young adult animals should be used. The eyes must be examined using fluorescein dye procedures at least 24 hours before application of the test substance. Animals showing preexisting corneal injury are to be eliminated.

(4) *Number of animals.* At least nine animals must be used.

(5) *Number and selection of dose.* A dose of 0.1 ml of liquid or 100 mg of solid must normally be applied to each test eye. Smaller quantities may be used when the standard quantities would be lethal, or when 100 mg of the solid cannot feasibly be administered to the eye.

(6) *Caging.* Caging must be designed to minimize exposure to sawdust, wood chips, and other extraneous materials that might enter the eye.

(b) *Study Conduct.* The test substance must be placed on the everted lower lid of one eye; the upper and lower lids are then to be gently held together for 1 second before releasing to prevent loss of material. The other eye, remaining untreated, serves as a control. The treated eyes of six rabbits must remain unwashed. The remaining three rabbits receive test material, and then the treated eye is flushed for one minute with lukewarm water starting no sooner than 20–30 seconds after instillation. A local anaesthetic to reduce pain in test animals may be used prior to administration of the test substance, provided that evidence can be presented indicating no significant difference in toxic reaction to the test substance will result from use of the anaesthetic.

(c) *Observation and scoring.* (i) *Observation.* Readings of ocular lesions must be made at 24, 48, and 72 hours after treatment and at 4 and 7 days after treatment. Readings must be made every 3 days thereafter, if injury persists, for at least 13 days after treatment or until all signs of reversible toxicity subside. Grading and scoring of irritation are to be performed in accordance with the following tables (from Draize, J. H., *et al.* (1965)). The most serious effects, such as pannus or blistering of the conjunctivae and other effects indicative of corrosive action must be reported separately.

(ii) Table of scale of weight scores for grading the severity of ocular lesions.

I. Cornea

(A) Opacity—Degree of Density (Area Taken for Reading) Scattered or diffuse area—details of iris clearly visible—1.

Easily discernible translucent areas, details of iris slightly obscured—2.

Opalescent areas, no details of iris visible, size of pupil barely discernible—3.

Opaque, iris invisible—4.

(B) Area of Cornea Involved.

One quarter (or less) but not zero—1.

Greater than one quarter—less than one-half—2.

Greater than one-half—less than three quarters—3.

Greater than three quarters up to whole area—4.

Score equals AXBX5 Total maximum = 80.

II. Iris

(A) *Values.*—Folds above normal, congestion, swelling, circumcorneal injection (any one or all of these or combination of any thereof), iris still reacting to light (sluggish reaction is positive)—1.

No reaction to light, hemorrhage; gross destruction (any one or all of these)–2.

Score AX5 total possible maximum = 10.

III. Conjunctivae

(A) Redness (Refers to Palpebral Conjunctivae Only).

Vessels definitely injected above normal—1. More diffuse, deeper crimson red, individual vessels not easily discernible—2.

Diffuse beffy red—3.

(B) *Chemosis.*—Any swelling above normal (includes nictitation membrane—1).

Obvious swelling with partial everson of the lids—2.

Swelling with lids about half closed—3.

Swelling with lids about half closed to completely closed—4.

(C) *Discharge.*—Any amount different from normal (does not include small amount observed in inner canthus of normal animals)—1.

Discharge with moistening of the lids and hairs just adjacent to the lids—2.

Discharge with moistening of the lids and considerable area around the eye—3.

Score (A+B+C)×2 Total maximum = 20.

The maximum total score is the sum of all scores obtained for the cornea, iris, and conjunctivae.

(d) *Data reporting and evaluation.* In addition to the information required by § 772.100–2(b)(8), Subpart A, the test report must include the following information:

(1) pH value of each test substance.

(2) In tabular form, the following data for each individual animal and the averages and range for each test group (eyes washed and unwashed):

(i) The primary eye irritation score at 24, 48, and 72 hours and 4 and 7 days and any other readings; and

(ii) Description of any serious lesions.

Appendix

(1) The following references provide specific information on protocols useful for evaluation of eye irritation.

(i) Committee for the Revision of NAS Publication 1138, Committee on Tox. Nat. Res. Council. 1977. Principles and Procedures for Evaluating the Toxicity of Household Substances. Pp. 1–9, 23–55. Prepared for the Consumer Product Safety Commission. Nat. Acad. Sci., Washington, D.C.

(ii) Draize, J. H., G. Woodard, and H. O. Calvery. 1944. Methods for the study of irritation and toxicity of substances applied topically to the skin and mucous membranes. J. Pharmacol. Exp. Ther. 83:377–390.

(iii) Draize, J. H. 1965. Appraisal of the Safety of Chemicals in Foods, Drugs, and Cosmetics—Dermal Toxicity, pp. 49–52. Assoc. of Food and Drug Officials of the U.S. Topeka, Kans.

(iv) Draize, J. H. 1959. The Appraisal of Chemicals in Foods, Drugs, and Costmetics, pp. 36–45. Association of Food and Drug Officials of the U.S. Austin, Tex.

(2) The following general references also provide good instruction on eye irritation studies:

(i) Federal Hazardous Substances Act Regulations. 16 U.S.C. 1500.

(ii) Loomis, T. A. 1974. Essentials of Toxicology. 2d Ed. Pp. 207–213. Lea & Febiger, Philadelphia.

§ 772.112–25 Primary dermal irritation study.

(a) *Study Design.* (1) *Condition of test substance.* (i) If the substance is a liquid, it must be applied undiluted.

(ii) If the test substance is a solid, it must be slightly moistened with physiological saline before application.

(2) *Species.* Testing must be performed in at least one mammalian species, preferably the albino rabbit. Selection of other species and strains may be acceptable, but must be justified.

(3) *Age.* Young adult animals must be used.

(4) *Number of Animals.* At least six animals must be used.

(5) *Number and selection of dose levels.* A dose of 0.5 ml of liquid or 0.5 g of solid or semisolid is to be applied to each application site.

(6) *Control groups.* (i) A vehicle control group is required if the vehicle is known to cause any toxic dermal reactions or if there is insufficient information about the dermal effects of the vehicle.

(ii) Separate animals are not required for an untreated control group. Each animal serves as its own control.

(b) *Study Conduct.* The test substance must be introduced under 1-inch square gauze patches. The patches must be applied to two intact and two abraded skin sites on each animal. In all animals, the application sites must be clipped free of hair. In addition, the abrasion must penetrate the stratum corneum, but not the dermis. A wrapping material such as gauze covered by an impervious, nonreactive rubberized or plastic material should be used to retard evaporation and keep the test substance in contact with the skin. The animals should be restrained. The test substance must be kept in contact with the skin for 24 hours. At the end of the exposure period, the wrapping should be removed and the skin wiped (but not washed) to remove any test substance still remaining. It may be necessary to rinse off the material if colored test substances are used.

(c) *Observation and scoring.* Animals must be observed and signs of erthyema and edema must be scored at 24 hours and 72 hours after application of the test substance. The irritation is to be scored according to the technique of Draize, J. H. (1959). Observation for irritation and scoring of any irritation must continue daily until all irritation subsides or is obviously irreversible.

(d) *Data reporting and evaluation.* In addition to the information required by § 772.100–2(b)(8), Subpart A, the test report must include the following information:

(1) pH value of each test substance.

(2) In tabular form, the following data for each individual animal and averages and ranges for each test group:

(i) Scores for erythema and edema at 24 hours, at 72 hours, and at any subsequent observations; and

(ii) Primary skin irritation scores according to the technique of Draize.

Appendix

(1) The following references provide specific information on protocols useful for evaluation of dermal irritation:

(i) Draize, J. H., G. Woodward, and H. O. Calvery. 1944. Methods for the study of irritation and toxicity of substances applied typically to the skin and mucous membranes. J. Pharmacol. Exp. Ther. 83:377–390.

(ii) Draize, J. H. 1965. Appraisal of the Safety of Chemicals in Foods, Drugs, and Cosmetics-Dermal Toxicity, pp. 46–59. Assoc. of Food and Drug Officials of the United States. Topeka, Kans.

(iii) Draize, J. H. 1959. The Appraisal of Chemicals in Foods, Drugs, and Cosmetics, pp. 36–45. Association of Food and Drug Officials of the United States, Austin, Tex.

(iv) Marzulli, F. N., and H. I. Maibach. 1977. Dermato-Toxicology and Pharmacology (Advances in Modern Toxicology). Vol. 4. Hemisphere Publishing Corp.: Washington, D.C.

(2) The following general references also provide good instruction on dermal irritation studies:

(i) Committee for the Revision of NAS Publication 1138, Committee on Tox., Nat. Res. Council. 1977. Principles and Procedures for Evaluating the Toxicity of Household Substances. Pp. 1–9, 23–59. Prepared for the Consumer Product Safety Commission, National Acad. Sci.: Washington, D.C.

(ii) Federal Hazardous Substances Act Regulations. 16 U.S.C. 1500.

(iii) Loomis, T. A. 1974. Essentials of Toxicology. 2d ed., pp. 207–213. Lea & Febiger. Philadelphia.

§ 772.112–26 Dermal sensitization study.

(a) *Study Design.* (1) *Condition of test substance.* The test substance must be applied undiluted. If the test substance causes marked irritation, it must be diluted with physiological saline until a concentration is found which produces only slight irritation. If the test substance is a solid to be injected intradermally, it should be dissolved in a minimum amount of physiological saline.

(2) *Species.* The test must be performed in at least one mammalian species. The albino guinea pig is the preferred species.

(3) *Age and sex.* Young adult males should be used when albino guinea pigs are tested. Young adults of either sex may be used when albino rabbits are tested.

(4) *Number of animals.* At least 10 animals must be used.

(5) *Number and selection of dose levels.* (i) An initial dose of 0.05 ml must be injected intradermally. This dose must be followed by injection of 0.1 ml three times weekly on alternate days for 3 weeks, so that a total of 10 treatments is administered. Following the 10th sensitizing treatment, the animals should be set aside for 2 weeks after which they should be challenged by a final injection (Landsteiner and Jacobs, 1935).

(ii) If the intradermal injection is impractical because the substance is highly irritating or cannot be dissolved or suspended in a form allowing injection, topical patch application can be substituted using the same schedule but 0.5 ml per application. For patch applications, other materials such as

water or alcohol can be used to moisten the test substance (see Buehler, E. V., 1965).

(6) *Controls.* (i) A positive control, using a known sensitizing agent, is recommended.

(ii) A concurrent vehicle control group is not required.

(b) *Study conduct.* (i) *Preparation of test animals.* Hair must be removed first by clipping and then by shaving from a strip running from flank to trunk along each side of each animal. This procedure must be repeated as necessary.

(ii) *Intradermal injection.* After preparation of the test animal, the test substance must be injected intradermally. The first sensitizing injection must be made by starting at one end of one strip. The succeeding injections must be made by moving along the shaved strip choosing a new location for each treatment.

(c) *Observation and scoring.* Erythema, edema, and other lesions must be scored at 24 hours and 48 hours after each application, according to the standard method (Draize, 1959).

(d) *Data reporting and evaluation.* In addition to the basic information required by § 772.100–2(b)(8), Subpart A, the following information must be reported:

(1) Tabular data for each animal on scores for erythema and edema at 24 and 48 hours postapplication or injection.

(2) Tabular data for the average score from all sensitizing treatments and the score of the challenge treatment.

Appendix

(1) Buehler, E. V. 1965. Delayed contact hypersensitivity in the guinea pig. Arch. Dermatol. 91:171–175.

(2) Committee for the Revision of NAS Publication 1138, Committee on Tox., Nat Res. Council. 1977. Principles and Procedures for Evaluating the Toxicity of Household Substances. Pp.1–9, 23–55. Prepared for the Consumer Product Safety Commission. Nat. Acad. Sci: Washington, D.C.

(3) Draize, J. H. 1959. The Appraisal of Chemicals in Foods, Drugs and Cosmetics. Association of the Food and Drug Officials of the U.S., Houston, Tex.

(4) Landsteiner, K., and J. Jacobs. 1935. Studies on sensitization of animals with simple chemical compounds. J. Exp. Med. 61:643–656.

(5) Marzulli, F. N., and H. I. Maibach. 1974. The use of graded concentration in studying skin sensitizers: experimental contact sensitization in men. Food Cosmet. Toxicol. 12(2):219–227.

(6) Marzulli, F. N., and H. I. Maibach. 1977. Dermato-Toxicology and Pharmacology (Advances in Modern Toxicology), vol. 4. Hemisphere Pub. Corp.: Washington, D.C.

(7) Schwartz, L. 1969. Twenty-two years experience in the performing of 200,000 prophetic patch test. Southern Med. Journal 53:478–483.

§ 772.112–31 Subchronic oral dosing studies.

(a) *Study Design.* (1) *Species.* Testing must be performed in at least two mammalian species, preferably the same species and strain for which chronic studies are anticipated. Once species must be a generally recognized strain of laboratory rat. The second species may be a nonrodent. The nonrodent species should usually be the dog. Selection of a nonrodent species other than the dog will require full and adequate justification which should consider such factors as the comparative metabolism of the chemical and species sensitivity to the toxic effects of the test substance, as evidenced by the results of other studies.

(2) *Sex and age.* Equal numbers of males and females of each species and strain tested must be used. The tester must begin to dose as soon as possible after weaning and environmental acclimatization but no later than six weeks of age for rodents and at 4–6 months of age for dogs.

(3) *Control group.* A concurrent control group is required. This group must be an untreated control group or, if a vehicle is used in administering the test substance, a vehicle control group. If the toxic properties of the vehicle are not known or cannot be made available, both untreated and vehicle control groups are required.

(4) *Number of animals.* Each test group and concurrent control group must contain at least 20 animals of each sex in studies with rats and at least 6 of each sex in studies with nonrodents. This number must be increased by the number, if any, scheduled to be sacrificed before completion of the study, such as, for example, rats on which hematology and blood chemistry determinations are made before and during the study.

(5) *Duration of testing.* (i) In studies with rodents, the substance being tested must be administered for at least 90 days.

(ii) In studies with nonrodents, the substance being tested must be administered daily for at least 6 months.

(6) *Number of dose levels and dose selection.* (i) At least three dose level groups (in addition to the control groups) must be tested.

(ii) The highest dosage level must result in toxicological or pharmacological effects, but not cause more than 10 percent fatalities. This level should be higher than that expected for human exposure.

(iii) The lowest dosage level must be one which does not induce any evidence of toxicity.

(7) *Route of administration.* The test substance must be administered in the animal's diet. Oral intubation may be allowed if the physical characteristics of the test substance so dictate. The chosen method must be used for all levels. If the test substance is administered by oral intubation, the amount of test substance must be adjusted weekly or biweekly to maintain a constant dose level in mg/kg (body weight). If the test substance is administered in the diet, either a constant concentration (ppm) or a constant dose level in mg/kg (body weight) must be used. The selection of dosage units of administration in the diet must be consistent with that for chronic feeding studies (Section 772.113–3 Subpart D).

(b) *Study Conduct.* (1) *Observation of animals.* All toxicological and pharmacological signs shall be recorded daily, including their time of onset, intensity, and duration. Such signs include but are not limited to: Mortality; and cardiovascular, respiratory, excretory, behavioral, and central nervous system (paralysis, ataxia, and pupillary reaction) effects. Observations must be made by an appropriately trained observer. Food consumption must be measured weekly during the test, and the animals must be weighed at least weekly. The animals must be observed as specified in Subpart A, § 772.100–2(b)(6)(ii). A complete ophthalmalogical examination must be conducted by a veterinarian on all nonrodents at the termination of the study.

(2) *Clinical laboratory testing.* The following determinations must be made at the time indicated below for each type of testing. For rodents, these determinations must be made on at least 10 animals of each sex in each group. For nonrodents, these determinations must be made on all animals in each group. Depending on the techniques used, it may be necessary to sacrifice animals to make the required clinical determinations. In case of said sacrifice, additional animals must be added to the study as provided by paragraph (a)(4) of this section.

(i) *Hematology.* Hematology determinations must be made as follows: For nonrodents shortly before the beginning of dosing, at least every 30 days thereafter, and at the termination of the testing period; and for rodents, shortly before the beginning of dosing,

at an intermediate time, and at the termination of the testing period. The following hematology determinations must be made: Hematocrit, hemoglobin, erythrocyte count, total and differential leukocyte counts, platelet count, and, if signs of anemia are present, reticulocyte count.

(ii) *Blood chemistry.* Blood chemistry determinations must be performed as follows: For nonrodents, shortly before the beginning of dosing, at least every 30 days thereafter, and at the termination of the testing period; and for rodents, shortly before the beginning of dosing, at an intermediate time, and at the termination of the study. Nonrodents must be fasted for 1 day prior to obtaining blood samples. The following determinations must be made: Calcium, potassium, serum lactic dehydrogenase, serum glutamic pyruvic transaminase, serum glutamic oxaloacetic transaminase, glucose, blood urea nitrogen, direct and total bilirubin, serum alkaline phosphatase, total cholesterol, albumin, globulin, total protein, and such other determinations as may be necessary for adequate toxicological evaluation. The following determinations may also be useful: Chloride, uric acid, blood creatinine, and gamma-glutamyl transpeptidase.

(iii) *Cholinesterase inhibition tests.* If the test substance contains a carbamate, an organophosphate, or any chemical that produces acetyl cholinesterase inhibition, the enzyme activity for plasma and red blood cell must be monitored shortly before the beginning of dosing, at least twice during the study, and at the end of the study, and the enzyme activity for brain must be monitored at the termination of the study. In addition, when nonrodents are used, monitoring of the enzyme activity must be performed twice before the beginning of dosing. Additionally, serial determinations may be useful to provide data on time-course of development of inhibition, extent of inhibition, and recovery from inhibition (e.g., after removal from treated diet); the undertaking of such determinations should not, however, result in over-stress of the test animals.

(iv) *Urinalysis.* Urinalysis must be performed as follows: for rodents, at least once (at an intermediate time) during the testing period, and again at the termination of the testing period; and for nonrodents, shortly before the beginning of dosing, every 60 days thereafter, and at the termination of the test. Nonrodents must be fasted 1 day prior to collection of urine samples. Each animal must be evaluated individually. The urinalysis must include specific gravity or osmolarity, pH, protein, glucose, ketones, bilirubin, and urobilinogen, as well as microscopic examination of formed elements. Results of these determinations must be expressed in quantitative terms by appropriate grading scales.

(v) *Additional tests.* Depending on the known or suspected properties of the test substance, such other determinations as may be necessary for adequate toxicological evaluation must be performed.

(3) *Handling of moribund and dead animals.* (i) *Moribund animals.* Moribund animals must be sacrificed to lessen the likelihood of unobserved death and subsequent autolysis or cannibalism.

(ii) *Tissue loss and dead animals.* Requirements concerning tissue loss and the handling of dead animals are specified in Subpart A, § 772.100–2(b) (6) and (7), respectively.

(4) *Gross necropsy.* (i) The standards set for necropsy procedures in § 772.100–2(b)(7), Subpart A, must apply.

(ii) All test animals in the study must be subjected to gross necropsy, which must include examination of the external surface; all orifices; the cranial cavity; carcass; the external and cut surfaces of the brain and spinal cord; the thoracic, abdominal and pelvic cavities and their viscera; and the cervical tissues and organs.

(iii) In addition, the following organs must be weighed: Liver, kidneys, heart, gonads, and brain. Also, for nonrodents, thyroid (with parathyroid), adrenals, and pituitary must be weighed. Prior to being weighed, organs must be carefully dissected and properly trimmed to remove fat and other contiguous tissue in a uniform manner. They must be weighed as soon as possible after dissection to avoid drying.

(iv) The gross necropsy findings must be recorded and reported in accordance with paragraph (g)(3) of this section.

(v) Tissue samples must be preserved and held in accordance with § 772.110–1(j), Subpart B.

(5) *Histopathology examination.* (i) *General.* A histopathology examination shall be performed on the organs and tissues of all animals in accordance with this paragraph.

(ii) *Nonrodents.* The following organs and tissues, when present, of each test animal must be subjected to microscopic study: all gross lesions, brain (at least 3 levels from the forebrain, midbrain, and hindbrain), spinal cord (at least 2 levels), eye, pituitary, salivary gland, heart, thymus, thyroid with parathyroid, lungs with mainstem bronchi, trachea, esophagus, stomach, small and large intestines, adrenals, pancreas, liver, gall bladder, kidneys, urinary bladder, aorta, testes, prostate, ovaries, corpus and cervix uteri, spleen, a representative lymph node, bone (with marrow), skeletel muscle, skin, sciatic nerve, and mammary gland. Sites from which bone and lymph nodes are taken must be indicated.

(iii) *Rodents.* The following organs and tissues of each test animal must be subjected to microscopic study:

(A) *All animals in control and high dose groups.* All gross lesions, brain (at least 3 levels), eye, pituitary, salivary gland, heart, thymus, thyroid (with parathyroid), lungs with mainstem bronchi, trachea, esophagus, stomach, small and large intestines, adrenals, pancreas, liver, kidneys, urinary bladder, testes, prostate, ovaries, corpus and cervix uteri, spleen, bone (with marrow), and skeletel muscle. Section of bone (with marrow, when present) should be taken from sternebrae, vertebrae, or the tibio-femoral joint (the last will also include attached muscle).

(B) *All animals in intermediate and low dosage groups.* Liver, kidney, heart, any gross lesion, and any target organ either at the high dose or from laboratory tests or clinical observation at any treatment level.

(iv) Tissue and slide preparation and retention.

(A) The standards set forth in § 772.100–2(b)(7)(ii), Subpart A apply.

(B) Tissue samples, tissue blocks, and microscopic slides must be preserved and held in accordance with § 772.110–1(j), Subpart B.

(v) *Examiner.* The standards set forth in § 772.100–2(b)(1)(i), Subpart A apply.

(vi) *Records.* The histopathology findings must be recorded and reported as required by paragraph (c)(4) of this section.

(c) *Data reporting and evaluation.* In addition to the general reporting requirements of § 772.100–2(b)(8), Subpart A, a subchronic oral dosing study test report must contain the following information, presented in the format specified (unless adequate justification is supplied to present these data in another form):

(1) Animal records and clinical laboratory data. The following information must be arranged by test group (dose level and sex). All means must be accompanied by standard deviation.

(i) Significant time periods, for individual animals. In tabular form, data must be provided showing, for each animal:

(A) Its identification number;

(B) Whether it died by sacrifice, and if so, whether it was moribund before sacrifice;

(C) Its age at the beginning of the study;

(D) The week of the test when sacrifice occurred or the animal's death was noted; and

(E) Its age at death.

(ii) Variation from requirements, for individual animals. In tabular form, data must be provided showing, for each animal that was not subjected to gross necropsy and histopathology examination in accordance with requirements of this section:

(A) Its identification number;

(B) The manner of variation; and

(C) The reasons for failure to comply with the requirements of this section.

(iii) Toxic, pharmacologic, and behavioral effects for individual animals. In tabular form, data must be provided showing, for each animal:

(A) Its identification number;

(B) The date of observation of each sign of toxicity, pharmacological effect, or behavioral abnormality; and

(C) A description of the toxic sign, pharmacological effect, or behavioral abnormality. If such a response occurs repeatedly, it need be described only once and may thereafter be described by reference, with any variations noted as appropriate.

(iv) Toxic, pharmacologic, and behavioral effects for test animals. In tabular form, data must be provided showing, for each test group (dose level and sex):

(A) A list of each sign of toxicity, pharmacological effect, or behavioral abnormality affecting any animal in the test group;

(B) For each sign, effect, or abnormality, the number of animals affected;

(C) For each sign, effect, or abnormality, the median time from the beginning of the study to the first observation of such response; and

(D) The median age at death of animals not sacrificed.

(v) Food and body weight data, for individual animals. In tabular form, data must be provided showing, for each animal:

(A) Its identification number;

(B) Measured food consumption at weekly intervals throughout the test period; and

(C) Body weight measured weekly throughout the test period.

(vi) Food and body weight data, means. In tabular and graphic form, data must be provided showing, for each test group (dose level and sex):

(A) Mean measured food consumption at weekly intervals throughout the test period; and

(B) Mean body weight measured weekly during the test period.

(vii) Weekly survival and sacrifice data. In tabular form, data must be provided showing: the number of animals in each group which remained alive at the end of each 7-day interval, the number of animals in each group that were sacrificed or otherwise died during each 7-day interval, and the number that died by sacrifice and were moribund before sacrifice.

(viii) Clinical laboratory test protocol.

(A) The rationale for timing of the clinical laboratory tests, if different from the standards set forth in paragraph (d) of this section; and

(B) The method and rationale for selecting animals for the clinical laboratory tests.

(ix) Clinical laboratory testing, for each animal. In any appropriate form, data must be submitted showing, for each animal:

(A) Its identification number; and

(B) The results of any hematological, blood chemistry, cholinesterase inhibition, urinalysis, and other clinical laboratory tests performed.

(x) *Clinical laboratory testing, for each test group.* In any appropriate form, data must be submitted showing, for each test group (dose level and sex), the average of the results of each hematologic, blood chemical, cholinesterase inhibition, urinalysis, and other clinical laboratory test performed.

(2) *Gross necropsy.* For all means in the data required in this subparagraph, the standard deviation must be stated. The following test information, arranged by test groups (dose level and sex), must be supplied in tabular form:

(i) Data showing the identification number of any animal in which any gross abnormality or gross lesion was noted, and containing, for each such animal, a description of each gross abnormality (including measurements), and the date (if known) when it was first observed. Gross abnormalities observed repeatedly need by described only once and may thereafter be described by reference, with any variations noted, as necessary.

(ii) Data showing the number of animals in which any type of gross abnormality was observed.

(iii) Data showing, for each animal: Its identification number, weights of the organs listed under paragraph (b)(4) of this section, and corresponding organ-to-body weight ratios.

(iv) Data showing the mean weights of each type of organ listed under paragraph (b)(4) of this section, and mean organ-to-body weight ratios.

(3) *Histopathology data.* The following information must be arranged by test group (dose level and sex). All means must be accompanied by standard deviation. The number of data units on which a calculation is based must be reported for all percentages and means.

(i) *Descriptions of lesions, for each animal.* Data must be submitted in any appropriate form showing:

(A) For each animal, its identification number, and a complete description and diagnosis of every lesion in the animal. Nonneoplastic lesions which are observed frequently or which are common in both treated and control animals should be graded. (Descriptions of neoplasms may also include grading.) A commonly used scale such as ±, 1, 2, 3, and 4, for degrees ranging from very slight to extreme can be used, but other scales are acceptable. If known, the description and diagnosis should identify any lesion which caused the animal to be moribund or to die. The description and diagnosis must include the time of appearance (if any) for each lesion. Abnormalities observed repeatedly need to be described only once, and may subsequently be supplied by reference, with any individual variations noted as necessary.

(B) For each animal, a table or paragraph listing the tissues found to be normal.

(C) If a grading system is used, a description of the system.

(ii) *Counts and incidence of lesions, by test groups.* Data must be submitted in tabular form showing, for each test group:

(A) The number of animals at the start of the test, and the number of animals in which any lesion was found;

(B) The number of animals affected by each different type of lesion, the average grade of each type of lesion, the number of animals examined for each type of lesion, and the percentage of those animals examined which were affected by each type of lesion; and

(C) The number of each different type of lesion.

(iii) *Incidence of tumors.* If a tumor is observed in any animal, the report must include a complete description and diagnosis of each tumor as required in § 772.113–1(k)(2)(i)(D), Subpart D.

(4) *Evaluation of data.* An evaluation of the test results (including their statistical analysis), based on clinical findings, gross necropsy findings, and histopathology results, must be made and supplied. This submission must include an evaluation of the relationship, if any, between the

animals' exposure to the test substance and the incidence and severity of all abnormalities, including behavioral and clinical abnormalities, gross and histopathologic lesions, organ weight changes, effects on mortality, and any other toxic effects. The evaluation must also include dose-response curves for any toxic or pharmacologic effect which appear to be compound-related for the various groups, and a description of statistical methods.

Appendix

(1) Committee for the Revision of NAS Publication 1138, Committee on Tox., Nat. Res. Council. 1977. Principles and Procedures for Evaluating the Toxicity of Household Substances. Pp. 1–21, 74–85. Prepared for the Consumer Product Safety Commission. Nat. Acad. Sci.: Washington, D.C.

(2) The following publications contain additional useful information:

(i) Boyd, E. M. 1972. Predictive Toxicometrics. Chapters 14, 15, and 16. Bristrol, Scientechnia Publisher, Ltd. (dist. Williams and Wilkens Co.: Baltimore).

(ii) Draize, J. H. 1959. The Appraisal of Chemicals in Foods, Drugs, and Cosmetics, pp. 28–30. Association of Food and Drug Officials of the United States. Austin, Tex.

(iii) Hagan, E. G. 1965. Appraisal of the Safety of Chemicals. Pp. 17–25 *in* Appraisal of Chemicals in Foods, Drugs, and Cosmetics. Association of Food and Drug Officials of the United States. Topeka, Kansas.

§ 772.112–32 Subchronic 90-Day Dermal Toxicity Study.

(a) *Study Design.* (1) *Species.* The albino rabbit weighing between 2.3 and 3.0 kg is the animal of choice. Selection of other species may be acceptable but must be justified.

(2) *Sex and age.* Equal numbers of young adult animals of both sexes must be used.

(3) *Number of animals.* At least 10 animals per sex per dose level must be used.

(4) *Number and selection of dose levels.* (i) At least three dose levels (in addition to controls) must be tested.

(ii) The highest dose level must show toxicological or pharmacological effects, but not cause more than 10 percent fatalities. This level should be higher than that expected for human exposure. If the test substance shows severe irritancy at this level, it should be diluted to a concentration such that primary skin irritation will not interfere with assessment of systemic toxicity.

(iii) The lowest dose level must be one which does not induce any evidence of toxicity.

(iv) If a vehicle is used in administering the test substance, all animals must receive about the same volume of dosing solution (i.e., the combined volume of test substance and vehicle should be similar for all dose levels).

(5) *Control groups.* A concurrent control group is required. This group must be an untreated control group or, if a vehicle is used in administering the test substance, a vehicle control group. If the toxic properties of the vehicle are not known or cannot be made available, both untreated and vehicle control groups are required.

(b) *Study Conduct.* (1) *General.* The hair must be clipped from the dorsal surface of the animal once per week throughout the study. Applications of test material must be made on the clipped unabraded skin and cover an area equivalent to 10 percent of the body surface area. The treated skin must be covered with patches secured in place with surgical hypoallergenic adhesive tape. The trunk of the animal must be covered with impervious material.

(2) *Duration.* The subchronic dermal exposure must be carried out with one application of the test material on each of 5 days per week for 13 consecutive weeks. Occlusion must last 6 hours daily. Half of the animals from each test group must be sacrificed following the last day of dosing. The remaining animals must be observed daily for a 2-week period following treatment and then sacrificed.

(3) *Observations.* All toxicological and pharmacological signs must be recorded daily, including their time of onset, intensity, and duration. Such signs include but are not limited to: Mortality; and dermal, cardiovascular, respiratory, excretory, behavioral, and central nervous system (paralysis, ataxia, and pupillary reaction) effects. Dermal irritation readings according to the method of Draize (1965) must be taken daily for the first 3 weeks of treatment and weekly thereafter. Observations must be made by an appropriately trained observer immediately prior to the next application of test substance. Food consumption must be measured weekly during the test. The animals must be observed at least every 12 hours as specified in Subpart A, § 772.100–2(b)(6).

(4) *Clinical laboratory testing.* The following determinations must be made at the times indicated for each type of testing. These determinations must be made on at least 5 animals of each sex in each group.

(i) *Hematology.* The following hematology determinations must be made at the beginning (shortly before dosing), at an intermediate time, and at the end of the test: Hematocrit, hemoglobin, erythrocyte count, total differential leukocyte counts, platelet count, and, if signs of anemia are present, reticulocyte counts.

(ii) *Blood chemistry.* The following blood chemistry determinations must be made at the beginning (shortly before dosing), at an intermediate time, and at the end of the test: Calcium, potassium, serum glutamic oxaloacetic transaminase, glucose, blood urea nitrogen, direct and total bilirubin, total cholesterol, serum alkaline phosphates, albumin, globulin, total protein, and such other determinations as may be necessary for adequate toxicological evaluation. The following additional determinations may also be useful: Chloride, uric acid, blood creatinine, and gamma-glutamyl transpeptidase.

(iii) *Cholinesterase inhibition tests.* If the test substance contains a carbamate, an organophosphate, or any chemical that produces acetyl cholinesterase inhibition, the enzyme activity for plasma and red blood cell must be monitored twice immediately before the beginning of the dosing, at least twice during the study, and at the end of the study, and the enzyme activity for brain must be monitored at the termination of the study. Additionally, serial determinations may be useful to provide data on time-course of development of inhibition, extent of inhibition, and recovery from inhibition (e.g., after removal from treatment); the undertaking of such determinations should not, however, result in over-stress of test animals.

(iv) *Additional tests.* Depending on the known or suspected properties of the active ingredient or the test substance, such other determinations as may be necessary for adequate toxicological evaluation should be performed.

(5) *Body weights.* Body weights of individual animals must be recorded at the beginning of the test and weekly thereafter.

(6) *Handling of moribund and dead animals.* (i) *Moribund animals.* Moribund animals must be sacrificed to lessen the likelihood of unobserved death and subsequent autolysis or cannibalism.

(ii) *Tissue loss and dead animals.* Requirements concerning tissue loss and the handling of dead animals are specified in Subpart A, § 772.100–2(b) (6) and (7), respectively.

(7) *Gross necropsy.* (i) The standards for necropsy procedures in § 772.100–2(b)(7), Subparty A apply.

(ii) All animals in the study must be subjected to gross necropsy, which must include examination of the external surface; all orifices; brain and spinal

cord; the thoracic, abdominal and pelvic cavities and their viscera; and the cervical tissues and organs.

(iii) In addition, the following organs must be weighed: Liver, kidneys, heart, gonads, brain, thyroid (with parathyroid), adrenals, and pituitary. Prior to being weighed, organs must be carefully dissected and properly trimmed to remove fat and other contiguous tissue in a uniform manner. They must be weighed as soon as possible after dissection to avoid drying.

(iv) The gross necropsy findings must be recorded and reported in accordance with paragraph (c)(2) of this section.

(v) Tissue samples must be preserved and held in accordance with § 772.110–1(j).

(8) *Histopathology examination.* (i) *General.* A histopathology examination must be performed on the organs and tissues of all animals in accordance with this paragraph.

(ii) *Microscopic studies.* The following organs and tissues of each test animal must be subjected to microscopic study.

(A) *All animals in control and high dosage groups.* All lesions, brain (at least three levels from the forebrain, midbrain, hindbrain), eye, pituitary, salivary gland, heart, thymus, thyroid (with parathyroid), lungs with mainstem bronchi, esophagus, stomach, small and large intestines, adrenals, pancreas, liver, kidneys, testes, urinary bladder, ovaries, corpus and cervix uteri, spleen, a representative lymph node, bone (with marrow), skeletal muscle, mammary gland, and any other target tissue. Multiple sections of treated and untreated skin must be studied. Sites from which bone and lymph nodes are taken must be indicated.

(B) *All animals in intermediate and low dosage groups.* Liver, kidney, heart, any gross lesion, and any target organ observed at the high dose, or any target organ suggested from laboratory tests or clinical observations at any treatment level.

(iii) Tissue and slide preparation and retention.

(A) The standards set forth in § 772.100–2(b)(7) (ii) and (iii), Subpart A, apply.

(B) Tissue samples, tissue blocks, and microscopic slides must be preserved and held in accordance with § 772.110–1(j).

(iv) *Examiner.* The standards set forth in § 772.100–2(b)(1)(i), Subpart A, apply.

(v) *Records.* The histopathology findings must be recorded and reported as required by paragraph (c)(3) of this section.

(c) *Data reporting and evaluation.* In addition to information meeting the general reporting requirements of § 772.100–2(b)(8), Subpart A, a subchronic 90-day dermal toxicity study test report must contain the following information, supplied in the format specified:

(1) *Animal records and clinical laboratory data.* The following information must be arranged by test group (dose level and sex). All means must be accompanied by standard deviation.

(i) Significant time periods, for individual animals. In tabular form, data must be provided showing, for each animal:

(A) Its identification number;

(B) Whether it died by sacrifice, and if so, whether it was moribund before sacrifice;

(C) Its age at the beginning of study;

(D) The week of the test when sacrifice occurred or the animal's death was noted; and

(E) Its age at death.

(ii) Variation from requirements, for individual animals. In tabular form, data must be provided showing, for each animal that was not subjected to gross necropsy and histopathology examination in accordance with requirements of this section.

(A) Its identification number;

(B) The manner of variation; and

(C) The reasons for failure to comply with the requirements of this section.

(iii) Toxic, pharmacologic, and behavioral effects, for individual animals. In tabular form, data must be provided showing, for each animal:

(A) Its identification number;

(B) The date of observation of each sign of toxicity, pharmacological effect, or behavioral abnormality; and

(C) A description of the toxic sign, pharmacological effect, or behavioral abnormality. If such a response occurs repeatedly, it need be described only once and may thereafter be described by reference. For each animal, a report of local skin effects, graded according to the system described in Draize (1965) must be submitted.

(iv) Toxic, pharmacologic, and behavioral effects, for test groups. In tabular form data must be provided showing, for each test group (dose level and sex):

(A) A list of each sign of toxicity (including local skin effects, pharmacological effect, or behavioral abnormality affecting any animal in the test group;

(B) For each sign, effect, or abnormality, the number of animals showing such effect, sign, or abnormality;

(C) For each sign, effect, or abnormality, the median time from the beginning of the study to when such response was first observed; and

(D) The median age at death of animals not sacrificed.

(v) Food and body weight data, for individual animals. In tabular form, data must be provided showing, for each animal:

(A) Its identification number;

(B) Measured food consumption weekly throughout the test period; and

(C) Body weight measured weekly throughout the test period.

(vi) Food and body weight data, averages. In tabular and graphic form, data must be provided showing, for each test group (dose level and sex):

(A) Mean food consumption measured weekly throughout the test period; and

(B) Mean body weight measured weekly throughout the test period.

(vii) Weekly survival and sacrifice data. In tabular form, data must be provided showing: The number of animals in each group which remained alive at the end of each 7-day interval, the number of animals in each group that were sacrificed or otherwise died during each 7-day interval, and the number that died by sacrifice and were moribund before sacrifice.

(viii) Clinical laboratory test protocol. The method and rationale for selecting animals for the clinical laboratory tests.

(ix) Clinical laboratory testing, for each animal. In any appropriate form data must be submitted showing, for each animal;

(A) Its identification number; and

(B) The results of any hematologic, blood chemistry, cholinesterase inhibition, and other clinical laboratory tests performed.

(x) Clinical laboratory testing, for each test group. In any appropriate form, data must be submitted showing, for each test group (dose level and sex), the average of the results of each hematologic, blood chemistry, cholinesterase inhibition, and other clinical laboratory test performed.

(2) *Gross necropsy data.* For all means in the data required in this paragraph, the standard deviation must be stated. The following test information, arranged by test groups (dose level and sex), must be supplied in tabular form.

(i) Data showing the identication number of any animal in which any gross abnormality or gross lesion was noted, and containing, for each such animal, a description of each gross abnormality or gross lesion (including measurements), and the date (if known) when the gross abnormality or lesion was first observed. Gross abnormalities

or lesions observed repeatedly need be described only once and may thereafter be described by reference, with any variations noted, as necessary.

(ii) Data showing, for each test group, the number of animals in which each type of gross abnormality was observed.

(iii) Data showing, for each animal: Its identification number, weight of its organs listed under paragraph (b)(7) of this section, and corresponding organ-to-body weight ratios and organ-to-brain weight ratios.

(iv) Data showing the mean weights of each type of organ, listed under paragraph (b)(7) of this section, mean organ-to-body weight ratios, and mean organ-to-brain weight ratios.

(3) *Histopathology data.* The following information must be arranged by test group (dose level and sex): All means must be accompanied by standard deviation. The number of data units on which a calculation is based must be reported for all percentages and averages.

(i) Descriptions of lesions, for each animal. Data must be submitted in a appropriate form showing:

(A) For each animal, its identification number, and a complete description and diagnosis of every lesion in the animal. Nonneoplastic lesions which are observed frequently or which are common in both treated and control animals should be graded. (Descriptions of neoplasms may also include grading.) A commonly used scale such as ±, 1, 2, 3, and 4 for degrees ranging from very slight to extreme can be used, but others are acceptable. If known, the description and diagnosis must include the time of appearance (if known) for each lesion. Abnormalities observed repeatedly need be described only once, and may subsequently be supplied by reference, with individual variations noted as necessary.

(B) For each animal, a paragraph listing the tissues examined and designation by check mark of those tissues found to be normal.

(C) If a grading system is used, a description of the system.

(ii) Counts and incidence of lesions by test groups. Data must be submitted in tabular form showing for each test group:

(A) The number of animals at the start of the test, the number of animals surviving to the termination of the test, and the number of animals in which any lesion was found;

(B) The number of animals affected by each different type of lesion, the average grade of each type of lesion, the number of animals examined for each type of lesion, and the percentage of those animals examined which were affected by each type of lesion; and

(C) The number of each different type of lesion.

(iii) *Incidence of tumors.* If a tumor is observed in any animal, the report must include a complete description and diagnosis of each tumor as required in § 772.113–1(k)(2)(i)(D), Subpart D.

(4) *Evaluation of data.* An evaluation of the test results (including their statistical analysis), based on clinical findings, gross necropsy findings, and histopathology results must be made and supplied. This submission must include an evaluation of the relationship, if any, between the animals' exposure to the test substance and the incidence and severity of all abnormalities, including behavioral and clinical abnormalities, gross histopathologic lesions, organ weight changes, effects on mortality, and other effects. The evaluation must include dose-resonse curves for any toxic or pharmacologic effect which appears to be compound-related for the various groups, and a description of statistical methods.

Appendix

Committee for the Revision of NAS Publication 1138, Committee on Tox., Nat. Res. Council. 1977. Principles and Procedures for Evaluating the Toxicity of Household Substances. Pp. 1–9,23–59, 74–85. Prepared for the Consumer Product Safety Commission. Nat. Acad. Sci.: Washington, D.C.

Draize, J. H. 1965. Appraisal of the Safety of Chemicals in Foods, Drugs, and Cosmetics. Association of Food and Drug Officials of the United States. Topeka, Kans.

§ 772.112–33 Subchronic inhalation toxicity study.

(a) *Study design.* (1) *Species and Age.* Testing must be performed on young adult laboratory rats.

(2) *Number and Sex of Test Animals.* A minimum of 10 animals per sex per exposure level must be used. This number must be increased by the number, if any, scheduled to be sacrificed before completion of the study, such as, for example, rats on which hematology and blood chemistry determinations are made before and during the study.

(3) *Number and selection of exposure concentration levels.* (i) At least three exposure concentration levels, in addition to the control(s), must be used.

(ii) The lowest atmospheric concentration must not show toxic effects.

(iii) The highest atmospheric concentration must demonstrate some toxicological effects, but not cause more than 10 percent fatalities. This level should be higher than that expected for human exposure.

(iv) All exposure levels and control(s) must be performed concurrently.

(4) *Duration of testing.* Animals must be exposed to the test substance at least 6 hours per day for at least 5 days per week over a 90-day period. Longer or more continuous exposures may be selected, depending on the test substance and the expected use pattern of the test substance. If shorter or less continuous exposures seem appropriate, the tester must consult with the Agency concerning the exposure times.

(5) *Use of vehicle.* A vehicle may be added to the test substance, if necessary, to help generate an exposure atmosphere. If the product's labeling instructions specify the use of a vehicle, that vehicle is preferred. If no vehicle is specified in the product's labeling instructions, the vehicle, if any, that has been used to formulate the product should be used, if possible.

(6) *Controls.* (i) *Vehicle control.* If any vehicle other than water is used in generating the exposure atmosphere, a concurrent solvent control group is required.

(ii) *Negative control.* A concurrent negative control group is required. These control animals must be treated in the same manner as all other test animals (including placement in exposure chambers), except that this control group must not be exposed to an atmosphere containing the test substance or any solvent.

(b) *Study conduct.* (1) *Exposure chamber design and operation.* Inhalation exposure techniques described in this section are based on the use of whole-body inhalation chambers. In such chambers, the experimental animals receive whole-body dermal exposure and possibly large oral exposure, as well as exposure by inhalation. In some cases, the tester may want to use other inhalation exposure techniques involving face masks, head-only exposures, intratracheal instillation, and other similar techniques which reduce or preclude dermal and oral exposures. Some alternative techniques are described by Phalen, 1976. When alternative techniques are used, the procedures and results must be reported in a manner similar to that required with the use of whole-body inhalation chambers.

(2) *Operational measurements.* The following measurements must be taken, with care to avoid major fluctuations in the air concentrations or major discrepancies in the operation of the chambers:

(i) *Air flow.* The rates of air flow through the chamber must be measured continuously.

(ii) *Chamber concentrations.* (A) Nominal concentrations must be calculated for each test exposure by dividing the amount of the agent used for the generating system by the air flow through the chamber during the exposure.

(B) Actual concentrations must be determined by samples of chamber air taken near the breathing zone of the animals as frequently as necessary to obtain an averaged integrated external exposure which is representative of the entire exposure period. The system used to generate the vapor, gas, aerosol must be such that the chamber concentrations are controlled under stable conditions, reflecting the current state-of-the-art, and must not vary in a range greater than 30 percent of the average (range/mean equal to or less than 30 percent).

(iii) *Temperature and Humidity.* The temperature must be maintained at $24 \pm 2°$ C and the humidity within the chamber at 40–60 percent. Both must be monitored continuously.

(iv) *Oxygen.* The rate of air flow through the chamber must be adjusted to insure that the oxygen content of the exposure atmosphere is at least 19 percent.

(v) *Particle size measurements.*

(A) *General.* In the case of gases and vapors, particle size measurements must be carried out at intervals to insure the animals are not being exposed to unknown and unexpected materials. Aerosol particle size measurements must be made on samples taken at the breathing level of the animals. These analyses must be carried out using techniques and equipment reflective of the state-of-the-art. All of the suspended aerosol (on a gravimetric basis) must be accounted for, even when most of the aerosol is not respirable.

(b) *Sizing analysis.* The sizing analysis must be in terms of equivalent aerodynamic diameters and must be represented as geometric mean (median) diameters and their geometric standard deviation (see NIOSH syllabus for reference) as calculated from log-probability graphs or computer programs. The size analyses must be carried out frequently during the development of the generating system to insure proper stability of aerosol particles and only as often thereafter during the exposure as is necessary to determine adequately the consistency of particle distributions to which the animals are exposed. At a minimum, these analyses must be carried out a daily basis.

(3) *Observation of animals.* All toxicological and pharmacological signs must be recorded daily, including their time of onset, intensity, and duration. Observations must be made at least 12 hours throughout the test period and, in particular, at the times the animals are exposed to the test substance. (Also see Subpart A, § 772.100–2(b)(6).) Such signs include, but are not limited to: Mortality; and cardiovascular, respiratory, excretory, behavioral, and central nervous system (paralysis, ataxia, and pupillary reaction) effects. Observations must be made by an appropriately trained observer. Food consumption must be measured weekly during the test, and the animals must be weighed at least weekly. Surveillance of animals must be made according to the requirements stated in Subpart A, § 772.100–2(b)(6).

(4) *Clincial laboratory testing.* The following determinations must be made at the times indicated below for each type of testing. These determinations must be made on at least five animals of each sex in each group. Depending on the technique used, it may be necessary to sacrifice animals to make the required clinical determinations. In case of such sacrifice, the number of animals started in the study must be increased by the number scheduled or anticipated to be killed before the end of the study.

(i) *Hematology.* The following hematology determinations must be made on at least five animals of each sex in each group at the beginning (before dosing), at an intermediate time, and at the termination of the testing period: Hematocrit, hemoglobin, erythrocyte count, total and differential leukocyte counts, platelet count, and if signs of anemia are present, reticulocyte count.

(ii) *Blood Chemistry.* Blood chemistry determinations must be performed at the beginning (before dosing), at an intermediate time, and at the termination of the study. The following determinations must be made: Calcium, potassium, serum lactic dehydrogenase, serum glutamic pyruvic transaminases, serum glutamic oxaloacetic transaminase, glucose, blood urea nitrogen, direct and total bilirubin, total cholesterol, serum alkaline phosphatase, albumin globulin, total protein, and such other determinations as may be necessary for adequate toxicological evaluation. The following additional determinations may also be useful: Chloride, uric acid, blood creatinine; and gammaglutamyl transpeptidase.

(iii) *Cholinesterase inhibition tests.* If the test substance contains a carbamate, an organophosphate, or any chemical that produces acetyl cholinesterase inhibition, the enzyme activity for plasma and red blood cell must be monitored twice before treatment, twice during treatment, and at the termination of the study, and the enzyme activity for brain at the termination of the study.

(iv) *Additional tests.* Additional tests such a blood pH, blood CO_2, specific enzyme analyses, and pulmonary function tests should be carried out in order to conform the diagnosis of suspected disease states or to help to follow the development of disease states known to occur with exposure to the test substance(s).

(5) *Handling of moribund and dead animals.* (i) *Moribund animals.* Moribund animals must be sacrificed to lessen the likelihood of unobserved death and subsequent autolysis or cannibalism.

(ii) *Tissue loss and dead animals.* Requirements concerning tissue loss and the handling of dead animals are specified in Subpart A, § 772.100–2(b) (6). and (7), respectively.

(6) *Gross necropsy.* (i) the standards set forth for necropsy procedures in § 772.100–2(b)(7)(i) must apply.

(ii) All animals in this study must be subjected to gross necropsy, which must include examination of the external surface; all orifices; the cranial cavity, carcass; the external and cut surfaces of the brain; spinal cord; the thoracic; abdominal, and pelvic cavities, and their viscera; and the cervical tissues and organs. The following organs and tissues must be examined for gross lesions: Adrenals, heart, lungs, trachea, bronchi, nasal passages and paranasal sinuses, spleen, liver, kidneys, stomach, small and large intestines, pancreas, ovary and uterus, testes with epididymis, prostate, urinary bladder, eye, bone (with marrow), and skin. Tissues in which any gross lesions are seen must be preserved for microscopic study.

(iii) Special treatment of the lung must be undertaken for morphological evaluation of the development of emphysema. Thus, the lungs must be removed *in toto,* weighed, and perfused intratracheally or intrabronchially (depending on the species) with an amount of 10 percent neutral buffered formalin that is equal to approximately 75 percent of the total lung capacity for that species. A maximum of 25 cc of water should be used for perfusion.

(iv) In addition, the organs which must be weighed include the brain, liver, kidneys, and heart. Prior to being weighed, organs must be carefully dissected and properly trimmed to remove fat and other contiguous tissue in a uniform manner. They must be

weighed as soon as possible after dissection to avoid drying.

(v) The gross necropsy findings must be recorded and reported in accordance with paragraph (c)(4) of this Section.

(vi) Tissue samples must be preserved and held in accordance with § 772.110–1(j).

(7) *Histopathology examination.* (i) To the extent indicated below in paragraphs (A) and (B), the following tissues must be examined microscopically:

(A) In the control and highest dose-level animals: Brain (at least 3 levels from the forebrain, midbrain, and hindbrain), eye, pituitary, salivary gland, thymus, heart, esophagus, lungs (with mainstem bronchi), trachea, nasal passages and paranasal sinuses, liver, stomach, small and large intestines, spleen, kidneys, thyroid (with parathyroid), adrenals, pancreas, urinary bladder, aorta, testes, ovaries, corpus and cervix uteri, bone (with marrow), skeletal muscle, skin, and all other tissues in which lesions were observed at necropsy; and

(B) In all other animals, the lungs, trachea, nasal passages and paranasal sinuses, liver, kidneys, and all tissues in which lesions were seen at necropsy and in which abnormalities were observed during the histopathology examination described in paragraph (b)(7)(i)(A) of this section.

(ii) Tissues and microscopic slides must be prepared according to the standards set forth in § 772.100–2(b)(7)(ii) and (iii), Subpart A. Tissue samples, tissue blocks, and microscopic slides must be preserved and held in accordance with § 772.110–1(j). A qualified pathologist must have final responsibility for the histopathology examination. The standards set forth in § 772.100–2(b)(1)(i), Subpart A, must apply.

(iv) The histopathology findings must be recorded and reported as required by paragraph (c)(5) of this section.

(c) *Data reporting and evaluation.* In addition to information meeting the general reporting requirements of § 772.100–2(b)(8), Subpart A, the test report must contain the following information, presented in the format specified:

(1) *Test conditions.* (i) *Chamber and generating system.* Description of the chamber design and operation including type of chamber, its dimensions, the source of make-up air and its conditioning (heating or cooling) for use in the chamber, the treatment of exhausted air, the housing and maintenance of the animals in the chambers, and similar related information. Equipment for measuring of temperature and humidity, the generating system, and the methods of analyzing airborne concentrations and particle sizing must be described.

(ii) *Exposure data.* The following chamber operational data must be tabulated individually and in summary form using means and standard deviations (with or without ranges) in tabular format. The data summaries must be grouped according to experimental groups, and the non-expected differences (such as temperature or airflow) tested for statistical significance.

(A) Airflow rates through the chamber;

(B) Chamber temperature and humidity;

(C) Nominal concentrations;

(D) Actual concentrations; and

(E) Median particle sizes and their geometric standard deviations.

(2) *Animal Records and Clinical Laboratory Data.* The following information must be arranged by test group (dose level and sex). All means must be accompanied by standard deviation.

(i) Significant Time Periods, for Individual Animals. In tabular form, data must be provided showing, for each animal:

(A) Its identification number;

(B) Whether it died by sacrifice, and if so, whether it was moribund before sacrifice;

(C) Its age at the beginning of study;

(D) The week of the test when sacrifice occurred or the animal's death was noted; and

(E) Its age at death.

(ii) Variation from Requirements, for Individual Animals. In tabular form, data must be provided showing, for each animal that was not subjected to gross necropsy and histopathology examination in accordance with requirements of this section:

(A) Its identification number;

(B) The manner of variation; and

(C) The reasons for failure to comply with the requirements of this section.

(iii) Toxic, Pharmacologic, and Behavioral Effects, for Individual Animals. In tabular form, data must be provided showing, for each animal:

(A) Its identification number;

(B) The date of observation of each sign of toxicity, pharmacological effect, or behavioral abnormality; and

(C) A description of the toxic sign, pharmacological effect, or behavioral abnormality. If such a response occurs repeatedly, it need be described only once and may thereafter be described by reference.

(iv) Toxic, Pharmacologic, and Behavioral Effects, for test Groups. In tabular form, data must be provided showing, for each test group (dose level and sex):

(A) A list of each sign of toxicity, pharmacological effect, or behavioral abnormality affecting any animal in the test group;

(B) For each sign, effect, or abnormality, the number of animals showing such effect, sign, or abnormality;

(C) For each sign, effect, or abnormality, the median time from the beginning of the study to when such response was first observed; and

(D) The median age at death of animals not sacrificed.

(v) Food and Body Weight Data, for Individual Animals. In tabular form, data must be provided showing, for each animal:

(A) Its identification number;

(B) Measured food consumption weekly throughout the test period;

(C) Body weight measured weekly throughout the test period.

(vi) *Food and body weight data, averages.* In tabular and graphic form, data must be provided showing, for each test group (dose level and sex):

(A) Mean measured food consumption weekly throughout the test period; and

(B) Mean body weight measured weekly throughout the test period.

(vii) *Weekly survival and sacrifice data.* In tabular form, data must be provided showing: The number of animals in each group which remained alive at the end of each 7-day interval. the number of animals in each group that were sacrificed or otherwise died during each 7-day interval, and the number that died by sacrifice and were moribund before sacrifice.

(viii) *Clinical laboratory test protocol.*

(A) The rationale for the timing of clinical laboratory test, if different from the standards set forth in paragraph (b)(4) of this section; and

(B) The method and rationale for selecting animals for the clinical laboratory tests.

(ix) *Clinical laboratory testing for each animal.* In any appropriate form, data must be submitted showing, for each animal:

(A) Its identification number; and

(B) The results of any hematological, blood chemistry, cholinesterase inhibition, and other clinical laboratory tests performed.

(x) *Clinical laboratory testing, for each test group.* In any appropriate form, data must be submitted showing, for each test group (dose level and sex), the average of the results of each

hematologic, blood chemical, cholinesterase inhibition, and other clinical laboratory test performed.

(3) *Gross Necropsy data.* For all averages in the data required in this subparagraph, the standard deviation must be stated. The following test information, arranged by test groups (dose level and sex), must be supplied in tabular form:

(i) Data showing the identification number of any animal in which any gross abnormality was noted, and containing, for each such animal, a description of each gross abnormality (including measurements), and the date (if known) when it was first observed. Gross abnormalities observed repeatedly need be described only once and may thereafter be described by reference, with any variations noted, as necessary.

(ii) Data showing the number of animals in which any type of gross abnormality was observed.

(iii) Data showing, for each animal: Its identification number, weights of its organs listed under paragraph (b)(6)(ii) of this section and corresponding organ-to-body weight ratios.

(iv) Data showing the mean weights of each type of organ listed under paragraph (b)(6)(ii) of this section, and mean organ-to-body weight ratios.

(4) *Histopathology data.* The following information must be arranged by test group (dose level and sex). All means must be accompanied by standard deviation. The number of data units on which a calculation is based must be reported for all percentages and means.

(i) Description of Lesions, for each Animal. Data must be submitted in an appropriate form showing:

(A) For each animal, its identification number, and a complete description and diagnosis of every lesion in the animal. Non-neoplastic lesions which are observed frequently or which are common in both treated and control animals must be graded. (Descriptions of neoplasms may also include grading.) A commonly-used scale such as ±1, 2, 3, and 4 for degrees ranging from very slight to extreme can be used, but other scales are also acceptable. If known, the description and diagnosis must identify any lesion which caused the animal to be moribund or to die. The description and diagnosis must include the time of appearance (if known) for each lesion. Abnormalities observed repeatedly need be described only once, and may subsequently be supplied by reference, with any individual variations noted as necessary.

(B) For each animal, a paragraph listing the tissues examined and designation by check mark of those tissues found to be normal.

(C) If a grading system is used, a description of the system.

(ii) Counts and Incidence of Lesions, by Test Groups. Data must be submitted in tabular from showing, for each test group:

(A) The number of animals at the start of the test, the number of animals surviving to the termination of the test, and the number of animals in which any lesion was found;

(B) The number of animals affected by each different type of lesion, the average grade of each type of lesion, the numbers examined for each type of lesion, and the percentage of those animals examined which were affected by each type of lesion; and

(C) The number of each different type of lesion.

(iii) Incidence of Tumors. If a tumor is observed in any animal, the report must include a complete description and diagnosis of each tumor as required in Section 772.113–1(k)(2).

(5) *Evaluation of Data.* An evaluation of the test results (including their statistical analysis), based on clinical findings, gross necropsy findings, and histopathology results, must be made and supplied. This submission must include an evaluation of the relationship, if any, between the animals' exposure to the test substance and the incidence and severity of all abnormalities, including behavioral and clinical abnormalities, gross and histopathologic lesions, organ weight changes, effects on mortality, and any other toxic effects. The evaluation must also include dose-response curves for any toxic or pharmacological effect which appear to be compound-related for the various groups, and a description of statistical methods.

Appendix

See the Appendix to § 772.112–23 for suitable sources of information on inhalation toxicology, exposure systems, generating systems, sampling methods, pulmonary function testing, and data interpretation.

Subpart E—Mutagenic Effects

§ 772.114–1 General.

(a) *Scope.* The standards in this subpart are designed to develop data on mutagenic health effects of chemical substances and mixtures ("chemicals") subject to mutagenic effects test regulations under the Toxic Substances Control Act (TSCA) (P.L. 94–469, 90 Stat. 2003, 15 U.S.C. 2601 *et seq.*).

(b) *Purpose.* (1) The objective of mutagenic testing is to assess the potential hazard to human beings of chemical substances or mixtures due to their interaction with genetic mechanisms with resultant heritable change (mutation). A large body of knowledge in the area of genetics leads to the conclusion that an increase in expressed genetic diseases can occur in the human population as a result of mutagenic chemicals.

(2) Mutation is a universal biologic mechanism underlying genetic changes in all systems. Mutations are generally observed to be deleterious in reference to the normal environment for an organism. The usual target molecule, DNA, is chemically the same in all cellular organisms. Therefore, test results from a wide variety of cells and organisms are relevant to the assessment of the mutagenic potential of a chemical for humans. One or a few molecules reacting with germ cell DNA may cause a heritable lesion.

(3) This heritable genetic change can occur by a variety of genetic mechanisms. This genetic change can be classified under gene mutations and chromosomal aberrations. In addition to detection methods that utilize phenotypic or structural end points, the potential of chemical to induce primary DNA damage can be detected by measuring stimulation or inhibition of recombination or repair. The following paragraphs present details of genetic end points that must be assessed to determine the potential of a chemical to cause mutations. It is recognized that, in some cases, the underlying chemical mechanisms may be the same for gene mutations and chromosomal aberrations when DNA is the target.

(i) *Gene mutations.* (A) *Point mutations.* These are intralocus changes which include base pair substitutions (transitions and transversions), frame shift mutations, and small deletions and insertions (which are larger than frame shift mutations or base pair substitutions but smaller than the gene itself).

(B) *Small multilocus mutations.* These are usually deletions where a locus has been physically removed from the chromosome in combination with other loci in the adjacent chromosome region.

(ii) *Chromosomal mutations.* (A) *Numerical changes.* Of special concern are monosomy and trisomy, such as may be produced by nondisjunction; these changes are termed aneuploidy. Also of concern are euploidy changes, especially triploidy and tetraploidy.

(B) *Structural changes.* These are large deletions, duplications, inversions, and translocations which are produced by chromosomal breaks and/or

misreplication and/or misrecombination effects.

(iii) Primary DNA damage: Stimulation of recombination.

(A) Mitotic crossing over

(B) Gene conversion

(C) Sister-chromatid exchange

(iv) Primary DNA damage: Repair stimulation and inhibition

(A) Nonreplicative DNA synthesis

(B) Differential killing of DNA-repair-deficient organisms

(c) *Data reporting and evaluations.* In addition to the general information required by § 772.100–2(b)(8) and any specific information derived pursuant to the test standards of §§ 772.114–2, 772.114–3, and 772.114–4, the following information must be reported:

(1) The reasons for using a given mutagenic test;

(2) A complete description of the test performed, including the rationales for the selection of species, strains, and cell types used;

(3) A complete history of the strain or cell type used, as well as the methods used to maintain and monitor the genetic integrity of the organism or cell type;

(4) The spontaneous mutation frequency given by concurrent controls and, if possible, by historical controls from the same laboratory and literature citation;

(5) The nature and results of genetic tests employed for defining an observed mutant phenotype as a heritable genetic effect;

(6) The sensitivity of the test system to chemical induction of mutations presented by positive controls, historical data, and references;

(7) The sensitivity and reproducibility of the test method documented from past work in the laboratory and references;

(8) The rationale for selection of dosage levels;

(9) The dose-response tabulations and curves with accompanying tabulation of normal growth characteristics and toxic effects of the test substance;

(10) The mutation frequency, expressed both numerically and as percentages or fractions;

(11) Where appropriate for insect and mammalian tests, number of males and/or females exposed and their age; broods or litters observed; number with mutations per number of progeny matings;

(12) The statistical method used for analysis, and the reference for the method;

(13) The statistical considerations used for the selection of number of test organisms and cells, supported by the appropriate references as to the power of the individual test at the concentrations of compound under test and the number of organisms and cells used, compared to the background controls;

(14) All repeat tests, particularly of microbial or *in vitro* tests; and

(15) Photographs of microscope slides necessary to define chromosomal aberration observed in a specific test. (Slides must be retained in accordance with § 772.110–1(j).)

(d) *General standards for testing.* (1) Sample sizes must be sufficiently large to yield conclusions of adequate precision. Statistical analyses must be appropriate to the experimental design used and to the inquiries motivating the tests.

(2) Culture conditions for *in vitro* tests which may affect detection of mutants and give falsely high or low figures for reasons other than chemical induction must be avoided.

(3) Tests must be performed in all respects in a manner known to give positive results for a wide range of chemical mutagens.

(4) Dose-response curves, reproducibility of results within each test, and similarity of results in comparison with other tests in the same class of genetic end points, will indicate the validity of each test.

(5) The appropriate genetic test must be performed or the rationale presented to define the genetic characteristics of any observed mutations.

(6) When a specific methodology is referenced in §§ 772.114–2, 772.114–3, and 772.114–4, the tester must perform the test following that methodology with essential modifications only. The rationale for a deviation from the methodology must be reported. Discussion with the Agency is required prior to conducting a study other than in accordance with the required methodology.

(e) *Standards for metabolic activation.* (1) Chemicals are often nonmutagenic unless converted to an active mutagen by metabolic processing. The reverse can also occur. Therefore, an understanding of the toxicology of a compound is essential, and a metabolic activation system must be incorporated into any test system other than intact mammals and insects.

(2) The test substance must be tested both in the presence and the absence of mammalian tissue extracts (with appropriate cofactors) which have been demonstrated to convert a wide range of chemical "premutagens" (substances which are mutagenically inactive in the absence of the tissue extracts) to mutagenically active substances. Rat or mouse liver extracts are acceptable. The tissue must be preinduced for the relevant enzymatic activities when appropriate. The inducer should be effective for the class of compounds under test. Other tissue extracts should be used in addition to liver extracts when the principal site of metabolism of the test substance is known not to be the liver, or when other tissues, including plant tissue, are known to give positive results with chemicals structurally related to these chemicals.

(3) As standardized procedures become available, it will be advisable to test for the presence of mutagens in the urine (and other body fluids) of animals to which the test chemical has been administered. Steps should be taken to free conjugated substances (e.g., by glucuronidase treatment) prior to testing urine.

(4) Data from an *in vivo* host-mediated activation assay may be submitted in addition to data from *in vitro* activation systems. The data from the *in vivo* host-mediated assay does not alone satisfy the requirements for metabolic activation systems. The target cells in the *in vivo* host-medicated assay should be inserted into the host tissues or cavities, taking care to minimize host effects on the target cells and to maximize the interaction between the potential metabolic activation components with the indicator organisms.

§ 772.114–2 Test standards for detecting gene mutations.

(a) *Controls for tests for detecting gene mutations.* All assays must be run with concurrent positive and negative controls with the possible exception of the mouse specific locus test.

(1) *Positive controls.* Positive control compounds must be selected to demonstrate both the sensitivity of the indicator organism and the functioning of the metabolic activation system. Positive controls should also be selected to demonstrate the sensitivity of the indicator cells or organisms to a compound with chemical characteristics similar to those of the test substance. For instance, an alkylating agent should be used as a control for an expected alkylator, and an intercalating agent for a suspected intercalator. Where applicable, the positive control should be administered by the same route as the test substance.

(2) *Negative controls.* Both a solvent and nonsolvent negative control must also be included.

(b) *Detection of gene mutations in bacteria.* (1) *Choice or organisms.* The

bacteria used must include strains capable of detecting base pair substitutions (both transitions and transversions) and frameshift mutations. The known spectrum of chemical mutagens capable of being detected by the strains must be considered when selecting the strains. The strains must also be highly sensitive to a wide range of chemical mutagens. They may include strains whose cell wall, DNA repair, or other capabilities have been altered to increase sensitivity (Ames, 1975; McCann *et al.*, 1975). Although sensitive bacterial assays for forward mutations at specific loci or over some portion of the entire genome may also be appropriate, at the present time the most sensitive and best-characterized bacteria for mutagenic testing are those capable of indicating reverse mutations at specific loci. Highly volatile compounds and gases will require special techniques for testing.

(2) *Methodology.* (i) *General.* The test must be performed in all respects in a manner known to give positive results for a wide range of chemical mutagens at low concentrations. A metabolic activation system must be utilized. (See § 772.114–1(e)). The sensitivity and reproducibility of the metabolic activation systems and strains used must be evaluated both by reference to past work with the method and by the concurrent use of positive controls.

(ii) *Plate assays.* In general, a test substance should be tested both by a spot test, in which a concentration gradient of the chemical is established in agar, and by plate incorporation assays at various concentrations. The spot test may not be useful if the test substance is insoluble in water, since this will prevent diffusion from the spot. Test conditions should minimize the possible effects of extraneous nutrients, contamination by other bacteria, and high levels of spontaneous mutants.

(iii) *Liquid suspension assays.* A few chemicals (e.g., diethyl-nitrosamine and dimethyl-nitrosamine) will give positive results only in tests in which the test subsance, the bacteria, and the metabolic activation system are incubated together in liquid prior to plating, but not in a plate incorporation assay (Bartsch *et al.*, 1976). Thus, it may be useful to conduct tests in liquid suspension as well as on agar plates. This is particularly recommended if the agar plate test gives ambiguous results or evidence of killing, or if a bacterial DNA repair test gives a positive result while the plate mutagenesis test does not.

(3) *Doses.* (i) *Plate assay.* For testing by the plate incorporation assay, a wide range of test doses should be used. There are as yet no simple methods for accurately predicting the doses of a chemical which are toxic to the bacteria under the conditions of the plate incorporation assay. Some indication of toxicity can be obtained by carefully examining the background growth on the agar plates at different test concentrations, and by comparing the number of mutants with the expected spontaneous background number. The highest level should be one which inhibits growth of the bacteria, unless this is not possible due to limited solubility of the test substance. Other doses should span at least a 1,000-fold range downward from the highest dose, and should be separated by no more than a factor of five between successive doses. Doses in a narrow range should generate data for an adequate dose-response curve for any test substance giving a mutagenic response.

(ii) *Liquid suspension assay.* For testing in liquid suspension, the highest dose should cause killing of at least 50 percent of the bacteria under the conditions of the assay or be in the range of 25–50 milligrams/ml of suspension. The highest dose tested may be lower than this due to limited solubility of the test substance. Additional doses should be separated by no more than a factor of five between successive doses. Doses in a narrow range should generate data for an adequate dose-responsive curve for any test substance giving a mutagenic response.

(4) *Data reporting and evaluation.* Results in the liquid suspension test must be expressed both as "mutants per surviving cell" and as "mutants per initial cells."

(c) *Detection of gene mutations in eukaryotic micro-organisms.* (1) *Choice of organism.* The organisms chosen must include strains capable of detecting base pair substitutions (aboth transitions and transversions) and frameshift mutations occurring in the nuclear genetic material. Strains of organisms that are as sensitive as possible to the test substance must be employed. These might include, for example, strains of *Saccharomyces, Schizosaccharomyces, Neurospora,* and *Aspergillus* that have been developed for the detection of reverse mutations and, to a more limited extent, forward mutation. Strains will likely become available eventually which will have increased sensitivity to chemical mutagens by virtue of other genetic alteration such as DNA repair deficiency. Effort should be made to employ such strains when they are developed, in order to cover the widest possible spectrum of genetic alterations.

(2) *Methodology.* (i) *General.* The test must be performed in all respects in a manner known to give positive results for a wide range of chemical mutagents. The test must incorporate a metabolic activation system. (See § 772.114–1(e).) The sensitivity and reproducibility of the method used must be evaluated both by reference to past work with the method and by concurrent use of positive controls. When possible, a compound must be tested in a liquid suspension test as well as a spot test. Highly volatile or gaseous compounds will require special techniques for testing.

(ii) *Plate assay.* Plate incorporation assays and spot tests should employ a wide range of doses, because there is no way of accurately determining the toxicity of the test substance under the conditions of the assay. Consideration should be given to minor modification of existing protocols to enhance the sensitivity of the test for certain compounds (e.g., spotting the compound versus incorporation into top agar, or replicating onto selective media after treatment for a period which permitted growth versus plate testing directly on selective media).

(iii) *Liquid suspension assay.* For liquid suspension tests, a preliminary experiment must be run to determine the toxicity of the compound in terms of colony-forming ability. Based on the determined toxicity, the highest dose tested must be toxic and result in at least 50 percent killing. A wide range of doses must be tested. Doses in a narrow part of the range should generate data for an adequate dose-response curve for any test substance giving mutagenic response. Consideration should be given to minor modification of existing protocols to promote maximum sensitivity to tests, such as prolonged treatment up to 96 hours under conditions that encourage growth of cells and/or conidia.

(3) *Data reporting and evaluation.* Results in liquid suspension must be expressed as a frequency of mutants in the surviving population, and spot test results must be expressed as mutants per unit population plated.

(d) *Detection of gene mutations in insects.* (1) *Choice of organisms.* The organisms chosen must include species and strains of insects capable of detecting forward gene mutations and small deletions in a significant portion of the genome. Of the different assay systems available in insects, the classical sex-linked recessive lethal test in *Drosophila* has superior

discriminatory power for genetic effects, since a large number of genes are screened.

(2) *Methodology.* (i) *General.* The genetic test employed must be the most sensitive available that is capable of detecting a wide range of chemical mutagens at low concentration levels. The sex-linked recessive lethal test (Abrahamson and Lewis, 1971) is recommended, since it samples one fifth of the *Drosophila* genome. The route of exposure must be abdominal injection, inhalation, or by feeding, depending upon the chemical characteristics of the test substance.

(ii) *Dose levels.* The doses must include a toxic concentration, a subtoxic concentration, and a lowest detectable effect concentration, if possible.

(e) *Detection of gene mutations in somatic cells in culture.* A number of tests in mammalian somatic cells in culture are available in which specific locus effects may be detected in response to chemical exposure. (Shapiro *et al.*, 1972; Sato *et al.*, 1972; Chu, 1971).

(1) *Choice of cell systems.* The cell line used must have demonstrated sensitivity to chemical induction of specific-locus mutations by a variety of chemicals. The lines must be chosen for ease of cultivation, freedom from biological contaminants such as mycoplasmas, high and reproducible cloning efficiencies, definition of genetic detection loci, and relative karyotypic stability. The inherent capabilities of the test cells for metabolic activation of promutagens to active mutagens should also be considered, as well as the use of metabolic activation systems similar to those used with microorganisms.

(2) *Methodology.* (i) *General.* The test must be performed in all respects in a manner known to give positive results for a wide range of chemical mutagens. The sensitivity of the system, metabolic activation capability, and its reproducibility must be evaluated by reference to past work and by the concurrent use of positive controls. Culture conditions which may affect the detection of mutations and give falsely high or low figures for reasons other than chemical induction must be avoided. Definition of detected genetic loci studied and verification that the observed phenotypic changes are indeed genetic alterations must be presented.

(ii) *Doses.* A wide range of test doses must be used. The toxicity of the chemical to the cells must be determined. Dose response curves for cell inactivation and mutant induction must be determined.

(f) *Detection of gene mutations in mammals.* (1) *Choice of organisms.* One mammalian test (the specific locus test in mice) is available at the current time to detect specific gene mutations induced in the germ cells of mice exposed to a mutagen (Russell, 1951; Searle, 1975). The test requires large numbers of animals for marginal sensitivity, and laboratories with the necessary mouse colonies and expertise are limited. In addition, there is concern that not all types of mutagenic events, e.g., base-pair substitutions, can easily be detected. However, this test does have the great advantage of estimating germ cell risk to genes in response to defined dose levels by the appropriate exposure route for toxicological evaluation in an intact animal. Other tests using biochemical markers in intact mammals are in the developmental stage and should be considered when they become available.

(2) *Methodology.* The test must be designed to detect changes in specific genetic loci in response to exposure at various dose levels to chemical mutagens. The exposure route should be the same as the expected human exposure route.

§ 772.114–3 Test standards for detecting heritable chromosomal mutations.

(a) *Controls for tests for detecting chromosomal damage.* Concurrent vehicle controls must be performed. Where appropriate, blank controls must be performed. Normal growth curves must be determined. Positive controls must be performed using a compound known to produce chromosomal defects and, if possible, to be structurally and/or mechanistically related to the test substance. The positive control should be run at dose levels comparable to the test compound. Where applicable, the positive control must be administered by the same exposure route as the test substance.

(b) *Detection of heritable chromosomal damage by the in vivo cytogenetics test in mammals.* (1) *General.* Cytological techniques are available to detect chromosomal anomalies in mammals *in vivo.* These procedures can be classified into methodologies designed to detect damage expressed in germ cells during early embryogenesis (non-transmitted and transmitted) and in somatic tissue (acute and chronic effects). The somatic tissue is normally bone marrow; the testis is used for germinal cells (Cohen and Hirschhorm, 1971; and Lubs and Samuelson, 1976).

(2) *Methodology.* Mammals must be used. The route of administration of the test substance must correspond to the principal expected route of human exposure to the test substance, or a rationale must be presented for use of an alternate route. Dosage regimens may be acute or subacute, but the dose schedule rationale must be specified and should reflect the expected human exposure. The medium must be optimal for the tissue employed. Double blind reading of the slides must be performed, and the microscope slides must be coded by reference to each animal's identification number. The micronucleus test may be used in addition to the *in vivo* cytogenetics test.

(3) *Data reporting and evaluation.* In addition to the basic information required by § 772.100–2(b)(8) and § 772.114–1(c), the following information must be reported:

(i) Vernier settings for the microscope;

(ii) Number of normal cells and all aberrations in chromatids or chromosomes by the individual types of aberrations;

(iii) A definition of breakdown by the individual type of aberration, including chromatid and chromosomal breaks, and stable and unstable intra- and inter-chromatid and -chromosome rearrangements, accompanied by photographs; and

(iv) Identification of chromosomal gaps separately.

(c) *Detection of heritable chromosomal damage using insects.* (1) *General.* Because of the superior size and morphology of chromosomes in some cells of *Drosophila,* the study of chromosomes in this insect has many advantages over comparable studies with other organisms. In addition, genetic confirmation of results is relatively simple. The selection of the species and strain must be on the basis of known sensitivity to the chemical induction of chromosomal defects. The available types of tests for chemically-induced defects include those for chromosomal rearrangements such as reciprocal translocation and position-effect tests, X or Y chromosome loss, and half-translocation tests (Abrahamson and Lewis, 1971). *Drosophila* also possesses the capability of metabolically activating at least some premutagens in a manner similar to mammals (Vogel, 1975).

(2) *Methodology.* The test method must be capable of demonstrating a heritable chromosomal effect.

(3) *Data reporting and evaluation.* In addition to the general information required by § 772.100–2(b)(8) and the specific requirements of § 772.114–1(c), the following must be reported:

(i) The staining and mounting techniques;

(ii) The criteria for scoring;

(iii) Numerical scoring of chromosomes, rather than percentages or fractions;

(iv) Characterization of the potentially sensitive germ-cell stages; and

(v) Description of each type of chromosomal lesion accompanied by photographs used to define the types of lesions.

(d) *Detection of heritable chromosomal effects by the dominant lethal test in mammals.* (1) *General.* This test is though to reflect chromosomal aberrations, but, by definition, it does not always measure heritable chromosomal effects. Many chemicals which induce dominant lethality are known to induce heritable chromosomal aberrations (not measured in this test). The actual measure in the dominant lethal test is early fetal loss. The assumption is that chromosomal abnormalities produced in sperm may be responsible for the developmental errors leading to the death of the zygote.

(2) *Methodology.* The methodology of the test is described by Epstein (1972). Toxicological factors and modifications are discussed by Green and Springer (1975) and Green *et al.* (1975). Acute, subchronic, and chronic exposure by various routes must be performed in a manner consistent with expected human exposure. Dosing and mating schedules must be designed to allow a sampling of potential effects on all sperm cell stages through meiosis.

(e) *Detection of heritable chromosomal effects by the heritable translocation test in mammals.* (1) *General.* Unlike the cytogenetic and dominant lethal tests, the heritable translocation test in rodents and/or insects allows analysis of heritability of chromosomal damage, i.e., the observation of an explicit genetic effect chemically induced in a parental generation and observed in progeny generations. The rodent tests possess the added advantage of reflecting mammalian metabolism in an intact organism. Induced effects on germ cell stages must be sampled through meiosis.

(2) *Methodology.* The heritable translocation test must be performed in rodents. The test must be designed to estimate defined genetic effects in mammalian germ cells in response to exposure to chemicals over a wide dose range by an appropriate route of administration. Dosing and mating schedules must be designed to allow a sampling of sperm cell stages throughout meiosis. Parental males must be treated and their male progeny mated to determine their semi-sterility or sterility. Cytogenetic analysis must be performed on males which are identified as sterile or semisterile to confirm the underlying chromosomal abnormality.

§ 772.114–4 Test standards for detecting effects on DNA repair or recombination as an indicator of genetic damage.

(a) *Controls.* All considerations discussed in § 772.114–2(a) are applicable. For a DNA repair test, the negative control must be a compound which is toxic to the indicator cells but is not more toxic to the deficient cells than to the repair-competent cells.

(b) *Detection of genetic damage in bacteria by DNA repair.* (1) *General.* (i) When the DNA of a cell is damaged by a chemical mutagen, the cell will utilize its DNA repair enzymes to attempt to correct the damage. Cells which have reduced capability of repairing DNA may be more susceptible to the action of chemical mutagens, as detected by increased killing. For suspension test using DNA repair-deficient bacteria, the postive control should be similar in toxicity to the test substance.

(ii) The DNA repair test in bacteria determines if the test substance is more toxic to DNA repair-deficient cells than it is to DNA repair-competent cells. Such differential toxicity is taken as an indication that the chemical interacts with the DNA of the exposed cells to produce increased levels of genetic damage.

(2) *Choice of organisms.* Two bacterial strains, with no known genetic differences other than DNA repair capability, must be used. The strains selected must be known to be capable of indicating the activity of a wide range of chemical mutagens. The spectrum of chemical mutagens capable of being detected by the strains and procedures used must be reported.

(3) *Methodology.* (i) *Plate test.* A compound should be tested by spotting a quantity on an agar plate which has had a lawn of the indicator organisms spread over it. After a suitable incubation period, the zone of inhibition around the spot must be measured for each strain and compared for the DNA repair-competent and DNA repair-deficient strains. If no discrete zone of inhibition is seen with either strain, then the results of the test are not meaningful and the test substance should be tested in liquid suspension.

(ii) *Liquid suspension test.* The liquid suspension test must be performed by comparing the rates at which given concentrations of the test substance will kill each of the two indicator strains when incubated in liquid suspension. Conditions should be adjusted so that significant killing of the DNA repair-competent strain occurs, if this is possible. Methodology is discussed in Kelly *et al.* (1969) and Rosenkranz *et al.* (1976).

(iii) *Doses.* The dose levels of test substance used in the plate or suspension test must be adjusted so that significant toxicity to the DNA repair-competent strain is measured. In the plate test, this means that a zone of inhibition must be visible; in the suspension test, significant loss of cell viability must be measured. This may not be possible if the test substance is not toxic to the bacteria or if, in the plate test, it does not dissolve in and diffuse through the agar. The same dose levels must be used in exposing the DNA repair-competent strains as for the repair-deficient strains.

(c) *Detection of genetic damage in mammalian somathic cells in culture: unscheduled DNA synthesis.* (1) *General.* DNA damage induced by chemical treatment of a cell may be measured as an increase in unscheduled DNA synthesis which is an indication of increased DNA repair. Unrepaired or misrepaired alterations may result in gene mutations or in breaks or exchanges which can lead to deletion and/or duplication of larger gene sequences or to translocations which may affect gene function by position effects (Stich, 1970; Stolz *et al., 1974).*

(2) *Methodology.* (i) *General.* Primary or established cell cultures with normal repair function repair must be used. Standardized human cell strains from repositories are recommended. Controls should be performed to detect changes in scheduled DNA synthesis at appropriate sections in the experimental design. The media conditions must be optimal for measuring repair synthesis.

(ii) *Dose.* At least five dose levels must be used, and the time in the cycle of synchronous or non-proliferating cells at which exposure takes place must be given. The maximum test substance dose must induce toxicity, and the dosing period with the test substance must not be less than sixty minutes.

(3) *Data reporting and evaluation.* In addition to the general information required by § 772.100–2(b)(8) and § 772.114–1(c), the following information must be reported:

(i) The specific activity and concentration of the tritiated thymidine as well as its source, lost number, and purity; and

(ii) Details of culturing, maintenance of cells lines, and medium.

(d) *Detection of genetic damage in eukaryotic microorganisms.* (1) *General.* One can effectively study the chromosomes of eukaryotic microorganisms by employing classical

genetic methodologies which depend upon the behavior and interaction of specific markers placed judiciously within the genome. These methods have been developed over several decades and have applied in recent years to the study of induced genetic damage (Zimmerman, 1971, 1973, 1975; Brusick and Andrews, 1974).

(2) *Choice of organisms.* Diploid strains of yeasts that detect mitotic crossing-over and/or mitotic gene conversion must be used. Additionally, as appropriate strains are developed, monitoring for induced non-disjunction and other effects may be possible. Mitotic crossing-over must be detected in a strain of organism in which it is possible, by genetic means, to determine with reasonable certainty that reciprocal exchange of genetic information has occurred. Strains used to detect mitotic gene conversion must be capable of demonstrating by genetic means that gene conversion has indeed occurred rather than some other genetic phenomenon. Strains employed for genetic testing must be of proven sensitivity to a wide range of mutagens.

(3) *Methodology.* (i) *General.* In general, chemicals must be tested in liquid suspension tests. Spot tests for induced mitotic gene conversion are available and may also be used. Specific modifications of treatment protocols such as duration of treatment, temperature, pH, and treatment medium must be justified. Results from liquid suspension tests must be expressed as recombinants per surviving population, and results from the plate test must be expressed as recombinants per initial cell population. Highly volatile compounds and gases will require special techniques for testing. Some compounds may present solubility problems and will, therefore, require special consideration for selection of the most suitable solvent.

(ii) *Doses.* Test substances must be tested first under conditions simulating the actual genetic test in order to determine toxicity. Based on results from these tests, the highest dose level must be toxic. Lower treatment levels should cover a 1000-fold range downward from the highest dose, and should be separated by not more than a factor of 10 between successive doses. an adequate dose-response curve must be generated for any test substance giving a positive response.

(e) *Detection of genetic damage in mammalian cells in culture: sister chromatid exchange.* (1) *General.* Sister-chromatid exchange in mammalian cells in culture appears to be a valuable ancillary test for detecting genetic damage. Other tests for detecting potential genetic damage may be available where chromosomal effects are seen as the genetic end-point.

(2) *Methodology.* (i) *General.* Exponentially growing cells of mammals in culture must be treated with chemicals and allowed to go through a cell division prior to assay for sister-chromatid exchange. The cell system must be chosen for a stable karyotype, low stable background of spontaneous sister-chromatid exchanges, and known capability of responding to chemical induction of sister-chromatid exchange. Metabolic activation must be incorporated where the characteristics and compatibility of the system for activation allows.

(ii) *Doses.* The maximum dose level must cause some cell death. A minimum of three dose levels above the control must be tested.

Appendix

(1) *General references.* The following sources provide general background material for mutagenic testing. In addition, the Journal of Mutation Research is publishing a series on standardized mutation testing procedures (Sobels, F. J. 1975. Editorial, Mut. Res. 31:1).

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Subpart F—Teratogenic/Reproductive Health Effects

§ 772.116–1 General.

(a) *Scope and purpose.* The standards in this subpart are designed to develop data on teratogenic and reproductive health effects of chemical substances and mixtures ("chemicals") subject to teratogenic/reproductive health effects test regulations under the Toxic Substances Control Act (TSCA) (P.L. 94–469, 90 Stat. 2003, 15 U.S.C. 2601 *et seq.*). The EPA will use these data to assess the risk of teratogenic and reproductive health effects these chemicals may present to human health.

(b) *Applicability.* These standards apply to the development of teratogenic and reproductive health effects data from the testing of chemicals specified in Part 771 of this subchapter.

(c) *Definitions.* The definitions in Section 3 of the TSCA and the definitions in § 770.2 and § 772.110–1(a)(3), entitled "Good Laboratory Practice Standards for Health Effects," apply to Subpart F. In addition, the following definitions also apply to Subpart F:

(1) "Teratogenic" means the potential of a test substance to produce defects in progeny (offspring) resulting from perinatal exposure;

(2) "Reproductive effects" means impairment of reproduction;

(3) "Developmental effects" means adverse health effects during any or all phases of development and maturation following single, continuous, or repeated administration of a test substance.

(4) "Reproductive toxicity" means the total or accumulated toxicity of a test substance during any or all phases of conception, development, and maturation.

(d) *Good Laboratory Practice Standards for Teratogenic/Reproductive Health Effects.* The Good Laboratory Practice Standards for Health Effects in § 772.110–1, Subpart B, apply to Subpart F.

(e) *Specific personnel requirements.* The specific personnel requirements in Subpart A, § 772.100–2(b) apply to Subpart F.

(f) *Submission of study plan.* The sponsor(s) of a study must submit a detailed study plan to the Agency at least 90 days before the initiation of a "reproductive study." The requirement for study plan submission in Subpart A, § 772.100–2(b)(2), applies to the reproductive studies in Subpart F. Study plans for teratogenicity studies are not required prior to their initiation.

(g) *Test or control substance concentration.* Section 772.100–2(b)(3) of Subpart A, apply to Subpart C.

(h) *Dietary requirements.* The dietary requirements as specified in Appendix B, Subpart A, apply to Subpart F.

(i) *Contaminant analysis of feeds and/or vehicles.* The specific requirements as specified in Appendix B, Subpart A, apply to Subpart F.

§ 772.116–2 Teratogenic effects test standards.

(a) *Study design.* (1) *Species and strain.* Testing must be performed in at least two mammalian species. The rat, mouse, hamster, or rabbit are acceptable. Other species may be used if adequate justification is supplied. One species must be the same as the species used in the reproductive study. Strains with low fecundity must not be used. Historical teratogenic data for the specific strain tested must be submitted.

(2) *Sex and age.* All test and control animals must be young, mature, pregnant females of uniform age, size, and parity. Prima gravida females are preferred.

(3) *Control groups.* Concurrent control group(s) are required as follows:

(i) A positive control group is required, unless historical data from the laboratory performing the test are submitted which demonstrate that the strains of animals being used are sensitive to known teratogenic agents.

(ii) A vehicle control group is required if a vehicle is used in administering the test substance. In addition, if there are insufficient data on toxic properties of the vehicle used in administering the test substance, an untreated (negative) control group receiving a sham treatment (e.g., physiological saline) is also required.

(iii) If no vehicle is used in administering the test substance, a separate control group receiving a sham treatment (e.g., physiological saline) is required.

(4) *Number of animals.* Each test and control group must include 20 or more pregnant females for rat, mouse, and hamster, and at least 12 pregnant females for rabbit.

(5) *Duration of test and time of delivery.* (i) The test subtance must be administered daily beginning at, or before, the time of implantation and continuing through the period of major organogenesis. Exposure of each species must encompass the gestation period up to the day before term.

(ii) Fetuses must be delivered by cesarean section approximately 1 day prior to term.

(iii) Females (parents) must ordinarily be sacrificed at time of cesarean section unless conditions indicate earlier sacrifice as required by paragraph (ii) of this section.

(6) *Dosage.* (i) At least three dosage levels must be tested in addition to the control(s).

(ii) The highest dosage level must induce some fetal or maternal toxicity, as demonstrated by body weight reduction or other toxic signs, but not cause more than 10 percent maternal fatalities. This level must be higher than that expected for human exposure.

(iii) The intermediate dose(s) must be spaced logarithmically (or at some approximate comparable point) between the high and low dosage level and must induce some observable fetal effects attributable to the test substance, when possible.

(iv) The low dosage level must induce no observable adverse effects attributable to the test substance.

(v) The dose administered to each animal must be based on the individual animal's body weight on the first day of test substance administration.

(vi) Dosing must be scheduled at approximately the same time during the day.

(7) *Route of administration.* To the extent possible, route(s) of administration should be comparable to the expected or known routes of human exposure. The test rules in Part 771 will specify the route(s) to be employed for a particular chemical.

(b) *Study conduct.* (1) *Animal care.* Food and water must be provided *ad libitum.* Pregnant females must be provided nesting materials or justification for not providing such material must be submitted. Animals may be individually caged or group caged.

(2) *Observation.* (i) The requirements for observation of animals as specified in Subpart A, § 772.100–2(b)(6) apply to Subpart F. Each such observation must be made by an appropriately-trained observer, who must note and record behavioral abnormalities, and all clinical signs of toxicity, including mortality.

(ii) Any female showing signs of abortion or premature delivery must be sacrificed on the day such evidence is observed. These animals must be analyzed, and all observations reported separately.

(iii) Females must be weighed at the first day of test substance administration and at sacrifice.

(3) *Necropsy.* (i) Immediately after the female is sacrificed, the uterus must be excised and weighed, then examined for fetal resorption, number of live fetuses and number of dead or resorbed fetuses. The litter weight of live fetuses must be determined.

(ii) One-half of each litter must be examined for skeletal anomalies, and the remaining one-half of each litter must be examined for soft tissue anomalies.

(iii) External and soft tissue examination of the fetuses must be performed by or under the supervision of an individual experienced and suitably trained interatogenic studies. The sex of each fetus must be determined, if possible. Gross observations of the skeleton and external and internal organs must be made with the aid of a dissecting microscope (or other instrument providing similar magnification). The internal gross morphology must be examined by sectioning through soft tissues (using razor blade sectioning or comparable techniques).

(iv) The necropsy data must be recorded and reported in accordance with paragraph (3) of this section.

(v) Entire fetuses must be preserved and held in accordance with § 772.110–1(j), Subpart B.

(c) *Data reporting and evaluation.* In addition to the basic information required by § 772.100–2(b)(8), Subpart A, the final test report must include the following information, presented in the format specified:

(1) *Test protocol.* Rationale for selection of the species and strain used.

(i) Dose levels (expressed as mg/kg of body weight per day) administered, and the rationale for their selection; and the number of days of tests substance administration.

(ii) Route and method of administration utilized and the rationale for selection if other than oral intubation.

(iii) Positive control data or historical data from the laboratory performing the test which demonstrate the sensitivity of the strains being used.

(iv) Justification statement for not providing nesting materials for pregnanat females, if such materials were not provided.

(2) *Maternal data.* (i) The following information, arranged by test groups, must be supplied in tabular form:

(A) Data showing, for each animal:

(*1*) Its identification number;

(*2*) Its age (or approximate age) at the start of the test;

(*3*) Data of caesarian section and sacrifice;

(*4*) Body weight on first day of dosing;

(*5*) Its body weight at sacrifice (actual, and corrected (by subtracting gravid uterus));

(*6*) The body weight change based on the foregoing weight measurements; and

(*7*) Any signs of abortion or premature delivery.

(B) Data showing, for each dose level:

(*1*) The number of animals initially on study;

(*2*) The number and percent that died;

(*3*) The number and percent that were pregnant; and

(*4*) The average maternal body weight change.

(ii) The following test information must be supplied in any appropriate form: A description of all observed signs of toxicity accompanied by the animal's identification number, test group, and date(s) of observation.

(3) *Fetal data.* When an anomaly is difficult to describe, a photograph of it may be submitted. All means must be accompanied by standard deviation. The following information arranged by test group must be supplied in tabular form:

(i) Numerical data showing, for each litter:

(A) Identification numbers;

(B) Number and percent of live fetuses;

(C) Average live fetal weight;

(D) Number of each sex, if determined;

(E) Number and percent of dead and resorbed fetuses;

(F) Number of implantations; and

(G) Number and percent of fetuses with any soft tissue of skeletal abnormality.

(ii) Anomaly data showing, for each litter:

(A) Identification number(s);

(B) Number of fetuses examined by necropsy;

(C) Number and percent of fetuses having soft tissue anomalies;

(D) Number of fetuses examined for skeletal anomalies;

(E) Number and percent of fetuses having skeletal anomalies; and

(F) Incidence and a full description of each type of anomaly.

(iii) Cumulative data showing, for each dose level:

(A) Identification of the dose level group;

(B) Number of litters examined;

(C) Number of implantations per litter;

(D) Average number of live fetuses per litter;

(E) Average of live fetal weights;

(F) Percent of dead and resorbed fetuses per litter;

(G) Number and percent of fetuses bearing anomalies of each kind observed;

(H) Number and percent of fetuses bearing any anomaly;

(I) Number and percent of abnormal fetuses per litter; and

(J) Number and percent of litters having anomalous fetuses.

(4) *Evaluation.* The litter or dam is an accepted unit for evaluation. Data on individual fetuses with anomalies should also be considered.

(i) Evaluation of the results with respect to observed effects, must include:

(A) An evaluation of the relationship, if any, between exposure to the test substance and the anomalies and all other toxic signs observed; and

(B) An indication of the dosage level at which no toxic effects attributable to the test substance would appear.

(ii) Statistical analyses must be performed to assist in the reporting and evaluation of data. All statistical methods used should be identified by reference and/or fully described.

Appendix

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§ 772.116–3 Reproductive effects test standards.

(a) *Study design.* (1) *Species.* Testing must be performed in at least one mammalian species which may be the same as one of the two species used in the teratogenic effects study pursuant to § 772.116–2 of this subpart. The rat is preferred.

(2) *Number and sex of animals.* In testing with rodents, each dose and control group must contain enough females to produce approximately 20 litters (20 sampling units) at each breeding, assuming typical mating and fertility for the strain. At least 10 fertile males per dose in the first mating of the F generation must be used. Subsequently at least 10 males per dose level are required.

(3) *Number of doses and dose selection.* (i) At least three dose level groups, in addition to the control group, must be tested.

(ii) The highest dose level must produce an observable toxicological or pharmacological effect in the test animals, but not cause more than 10 percent fatalities. This level must be higher than that expected for human exposure.

(iii) The lowest dose level must produce no observable adverse effects.

(4) *Control group.* Concurrent control groups are required as follows:

(i) A vehicle control group is required if a vehicle is used in administering the test substance. If there are insufficient data on the toxic properties of the vehicle used in administering the test substance, a separate control group receiving no chemical treatment is also required.

(ii) If no vehicle is used in administering the test substance, a separate control group receiving a sham treatment (e.g., physiological saline) is required.

(5) *Route of administration.* To the extent possible, route(s) of administration should be comparable to the expected or known routes of human exposure. The test rules in Part 771 will specify the route(s) to be employed for a particular chemical.

(6) *Duration of testing.* (i) The test substance must be administered to two generations of animals, F_0 and F_1. A third generation of animals, F_2, will be exposed to the test substance *in utero* and through nursing.

(ii) Dosing of animals in the F_0 generation must begin as soon as possible after weaning and acclimatization, and in any case before the animals are 6 weeks old. The test substance must be administered daily to the F_0 generation. Dosing must continue until all F_1 generation animals have been weaned.

(iii) Dosing of the animals selected from the F_1 generation for breeding must begin as soon as the animals are weaned (approximately 30 days after birth). The test substance must be administered daily to these animals. dosing must continue until 30 days after all F_2 animals have been weaned. (Dosing of animals from F_1 generation is not required if they have not been selected for breeding).

(b) *Study conduct.* (1) *Breeding.* After the F_0 generation animals such as rodents and lagomorphs have received the test substance for at least 100 days, they must be bred to produce the F_1 generation. Appropriate numbers of males and females must be selected at random from different litters of the F_1 generation for breeding. After the test substance has been administered to these animals for at least 120 days, they must be bred to produce the F_2 generation. Figure 1 of the Appendix indicates an acceptable breeding and dosing schedule.

(2) *Animal care.* Pregnant females must be caged separately and furnished with nesting materials.

(3) *Observations.* The requirements for observation of animals as specified in Subpart A, § 772.100–2(b)(6) apply to Subpart F.

(i) *Frequency.* Each animal must be observed for effects as long as it is being exposed to the test substance. Animals must be observed as frequently as necessary to obtain the data required by paragraph (c) of this section and Subpart A, Section 772.100–2(b)(6).

(ii) *Growth and delivery data.* The weight of each weanling must be recorded weekly to weight maturity and monthly thereafter. The dates of delivery must be recorded.

(iii) *Maternal data.* Observation must be made of the general condition and behavior of mothers, including nesting and nursing. Any abnormalities must be recorded.

(iv) *Paternal data.* Measurments must be made of spermatogenesis of all males in the F_0, F_1, and F_2 generations used to produce the subsequent generations. Such measurements should be undertaken within one week after breeding. In addition, or as an alternative, histopathology examinations of the tests, as indicated in paragraph (c)(11) of this section, must be undertaken. Additional useful information may be obtained by histopathology examinations of the tests of males in the F_0 and F_2 generations. particularly those males used for producing the subsequent generations. If spermatogenesis or histopathology of tests is evaluated in males in the F_0 and F_2 generations, such males should be of the same approximate age and should have been dosed for the same approximate length of time as males used in the F_1 generation (at the time the F_1 generation males were examined).

(v) *Litter data.* All litters must be examined as soon as possible after delivery. Where possible, effort should be made to prevent cannibalism of young. The following must be recorded: Litter size; number of stillborn; and number of live births. Viability counts and pup weight must be recorded at birth, four days after birth, and weaning. Additional viability counts between the fourth day and weaning are required for non-rodents. Any physical or behavioral abnormalities must be recorded.

(4) *Gross necropsy and histopathology.* (i) *F_1 generation.* Ten males and 25 females from each dose level and the control group must be subjected to a complete gross necropsy

and histopathology examination. The animals must be chosen from the F_1 generation animals used to produce the F_2 generation. The animals must be sacrificed at the end of the required period of dosing. The necropsy and histopathology examination must include examination of the reproductive organs.

(ii) *F_1 and F_2 generation.* A complete gross necropsy and histopathology examination must be conducted on five randomly selected weanlings of each sex from each test group (dose level and sex) in each generation (F_1 and F_2).

(iii) *Conduct of examinations.* All examinations must be conducted by or under the supervision of a qualified pathologist. The standards set forth in § 772.100–2(b)(1) and (7), Subpart A Apply.

(c) *Data reporting and evaluation.* The tester must submit to EPA the following reports:

"Study Plan" as required in § 772.100–2(b)(2), Subpart A;

"Interim Quarterly Summary Reports" outlining the current status of the study including any significant findings; and a "Final Test Report".

In addition to the basic information required by § 772.100–2(b)(8), Subpart A, the "Final Test Report" must include the following information, presented in the format specified:

(1) *Test protocol.* (i) The rationale for species and strain selection; and

(ii) The rationale for selection for the dosage levels; dosage levels must be reported as mg/kg/day as well as ppm.

(2) *Animal data.* For all means in the data required in this subparagraph, such means must be accompanied by the standard deviation.

(i) *Female data.* The following information relating to the reproduction of each female must be supplied in tables, with footnotes and description where appropriate:

(A) For each animal: Date of delivery; and unusual or abnormal behavior during estrous, gestation, or delivery; and fertility.

(B) Cumulative data showing means for controls and each dose level group in the F_0 and F_1 generation: the gestation index; approximate duration of gestation; and number and percent of animals showing behavioral abnormalities in connection with reproductive activity.

(C) For each mother: Its identification number; any abnormalities in nesting or nursing: total number of offspring per litter; number and percent of live and dead offspring; and general condition of offspring and mother through weaning.

(D) For each dose level and control group in the F_0 and F_1 generation: The fertility index; average size of litter; average number of dead and live offspring per litter; and number and percent of mothers showing behavioral abnormalities in nesting and nursing.

(ii) *Male data.* For each male evaluated for spermatogenesis in accordance with paragraph (b)(3)(iv) of this section: identification number and the results of the evaluation.

(iii) *Litter data on preweanling animals.* The following litter data on preweanling animals must be supplied in tables, with footnotes and descriptions where appropriate:

(A) For each litter arranged by dose level and generation: Total litter size; number and percent of stillborn; number and percent of live births; viability index; lactation index; weekly viability counts and weekly weight of each pup from day 4 of life to weaning; and number and nature of physical abnormalities observed.

(B) For each dose level and generation: Mean weekly weight of all pups from day 4 of life to weaning; number and percent of pups with physical or behavioral abnormalities; number and percent of pups surviving at birth, 1 week, and 3 weeks; and mean viability and lactation indices.

(iv) *Litter data on postweanling and mature animals.* the following informaiton, arranged by test group (dose level and sex), must be supplied in tabular form (unless adequate justification is supplied to present these data in another form):

(A) For each animal: Its identification number; its age at the beginning of the study; its age at death and manner of death; and its weight, as measured weekly through 1 month of age and monthly thereafter.

(B) Cumulative data showing means for each control and test group: The weekly or monthly weights; and the number and percent of animals with behavioral abnormalities.

(3) *Gross necropsy data.* The following test information, arranged by test groups (dose level and sex) must be reported:

(i) Data showing the identification of any animal for which any gross abnormality or lesion was observed, and containing for each such animal a description of each abnormality or lesion. Gross abnormalities or lesions observed repeatedly in gross necropsies need be described only once and thereafter may be described by reference.

(ii) Data showing the number of animals affeced by each type of abnormality or lesion; and the number of animals in which any abnormality or lesion was observed.

(4) *Evaluation.* (i) Evaluation of the results with respect to all toxic or pharmacological effects, including:

(A) An evaluation of the relationships, if any, between exposures to the test substance and the incidence and severity of effects (including effects on reproduction, behavior, tumors and lesions, and mortality).

(B) An indication of the dosage level at which no toxic effects attributable to the test substance would appear.

(ii) Statistical analyses must be performed to assist in the reporting and evaluation of data. All statistical methods used must be identified by reference and/or fully described.

Appendix

(1) Figure 1. This figure indicates an acceptable breeding and dosing schedule for the reproduction study described in § 772.116–3.

(2) Definitions of reproductive indices:

(i) "Fertility index" means the percentage of mating resulting in pregnancy.

(ii) "Gestation index" means the percentage of pregnancies resulting in the birth or live litters.

(iii) "Viability index" means the percentage of animals born that survived 4 days or longer.

(iv) "Lactation index" means the percentage of animals alive at 4 days that survived the 21-day lactation period.

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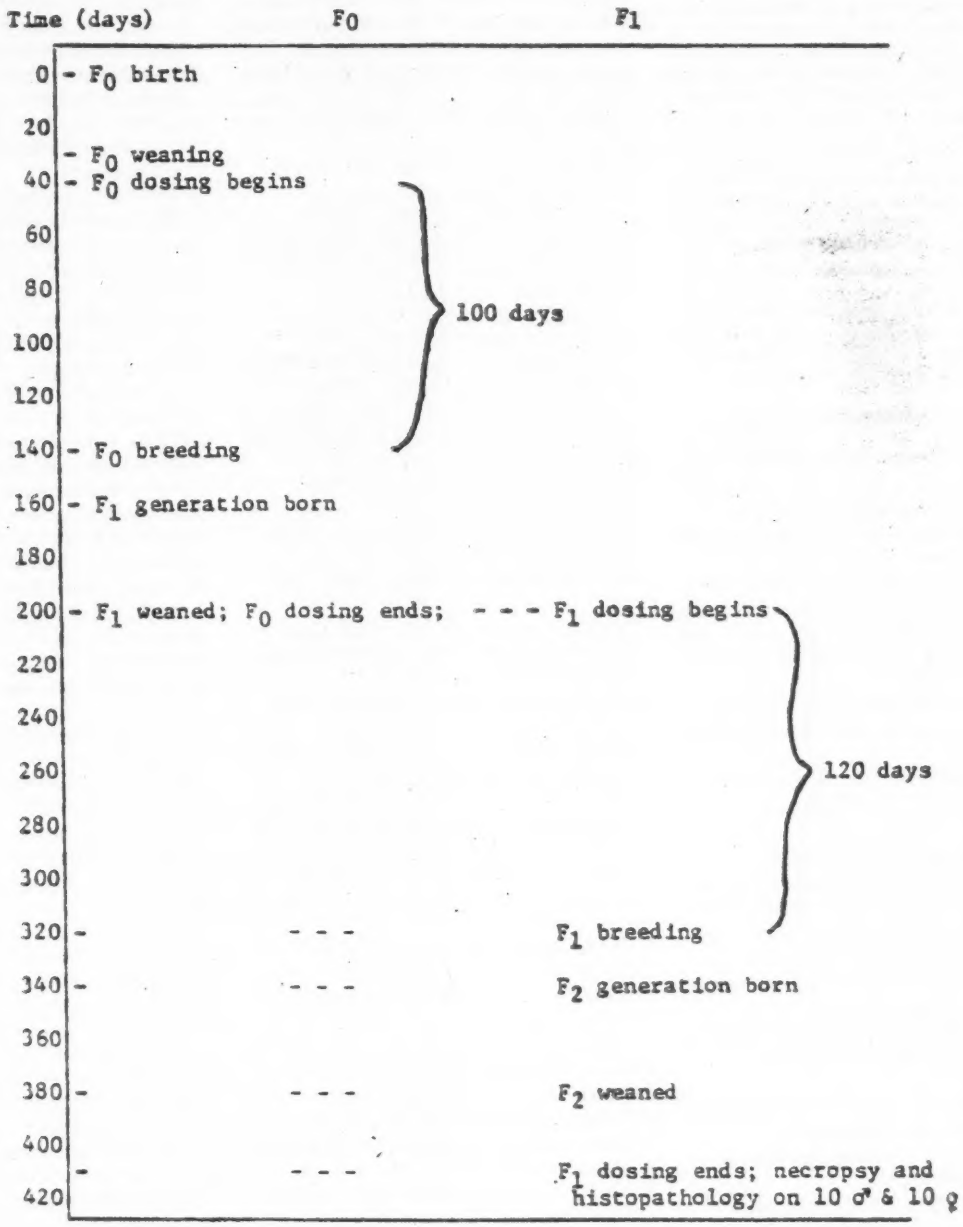
Figure 1. Approximate breeding and dosing schedule for Reproduction Study



F_0 total dosing ∿ 100 days	F_1 total dosing ∿ 210 days	F_2 total dosing 0 days
F_0 total exposure ∿ 160 days	F_1 total exposure ∿ 270 days	F_2 total exposure ∿ 60 days

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(3) References:

(i) Committee for the Revision of NAS Publication 1138, Committee on Tox., Nat. Res. Council. 1977. Principles and Procedures for Evaluating the Toxicity of Household Substances. Pp. 1–9, 99–110. Prepared for the Consumer Prod. Safety Commission. Nat. Acad. Sci.: Washington, D.C.

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Subpart I—Other Health Effects

§ 772.119–1 General Metabolism Test Standards.

(a) *Scope.* The standards in this subpart are designed to develop data on metabolic effects of chemical substances and mixtures ("chemicals") subject to health effects test regulations under the Toxic Substances Control Act (TSCA), (Pub. L. 94–469, 90 Stat. 2003, 15 U.S.C. 2601 et seq.).

(b) *Purpose.* A general metabolism study is required for the following major purposes:

(1) To identify and, to the extent possible, quantify significant metabolites;

(2) To determine any possible bioaccumulation and/or bioretention of the test substance and/or metabolites;

(3) To determine test substance absorption as a function of dose;

(4) To characterize route(s) and rate(s) of test substance excretion;

(5) To relate test substance absorption to the duration of exposure of the animals; and

(6) To obtain an estimate of binding of the test substance and/or its metabolites by target macromolecules in potential target organs.

(c) *Applicability.* These standards apply to the development of health effects data from the testing of chemicals specified in Part 771 of this subchapter.

(d) *Definitions.* The definitions in section 3 of the TSCA and the definitions in § 770.2 and § 772.110–1(a)(3), entitled "Good Laboratory Practice Standards for Health Effects," apply to Subpart I.

(e) *Study Design.* (1) *Substances to be tested.* Single does testing must be performed with the radioactively labeled form of the test substance. If a radioactively labeled form cannot be used, consultation with the Agency is required. The label, preferably ^{14}C, must be positioned at a site in the molecule which is not subject to enzymatic attack. Labeling with ^{3}H by the wilzbach procedure is unsatisfactory. In addition, some animals must receive repetitive doses of nonradioactively labeled test substance.

(2) *Test animal.* (i) *Species.* Testing must be performed in at least one mammalian species, preferably the laboratory rat of the same strain used in the chronic study (§ 772.113–3) or the oncogenicity study (§ 772.113–2).

(ii) *Age and sex.* Young adult male and female animals must be used.

(iii) *Number.* At least five animals per sex per test group must be tested.

(3) *Routes of administration.* The oral route (intubation) is required for dosing all radioactive forms, except for the single intravenous administration described for Group A in paragraph (f)(1)(i) of this section. Nonradioactive test substance may be given either by oral intubation or incorporated in the diet.

(4) *Dose levels.* At least two dose levels are required. The high dose level must produce some toxic or pharmacologic sign, and the low dose level must produce no toxic or pharmacologic sign, as demonstrated by an acute oral toxicity study.

(f) *Study conduct.* (1) The following four groups of animals must be studied.

(i) Group A animals must each receive only a single intravenous dose of the radioactively labeled test substance at the low dose level.

(ii) Group B animals must each receive only a single oral dose of radioactively labeled test substance at the low dose level.

(iii) Group C animals must each receive a series of daily oral doses of the nonradioactively labeled test substance (either by intubation or incorporated in the diet) over a period of at least 2 weeks, followed within 24 hours by a single oral intubation of the radioactively labeled test substance, each dose consisting of the low dose level.

(iv) Group D animals must each receive only a single oral dose of radioactively labeled test substance at the high dose level.

(2) *Time period and caging.* The animals in each group must be kept in individual metabolism cages for 7 days after the radioactive dose or until 95 percent of the administered dose is excreted (whichever occurs first), at which time the animals must be killed.

(3) *Analysis.* (i) *Levels of radioactivity.* (A) Levels of radioactivity in blood, urine, feces, and expired air must be measured at appropriate intervals throughout the study for all groups. However, if a preliminary study shows that no volatile radioactive materials are exhaled during the period of zero to 24 hours after dosing, such evidence may be submitted in lieu of measuring radioactivity of expired air for this study.

(B) For all groups, levels of radioactivity in tissues and organs must be measured at sacrifice by combustion to $^{14}CO_2$, with particular attention to bone, brain, fat, gonads, heart, kidney, liver, muscle, spleen, any tissue or organ which demonstrated pathology, and residual carcass.

(C) Samples of tissues and organs obtained at sacrifice, for all groups, must be extracted by nonpolar and polar solvents, and these extracts assayed for level of radioactivity. Extracted tissue residues can be combusted to $^{14}CO_2$ or solubilized with an appropriate solubilizing agent to measure the residual radioactivity.

(ii) *Chemical assay.* Urine and extracts of feces from all groups must be subjected to thin-layer chromatography and radiochromatogram scanning (or similar technique) for a determination of the extent of test substance biotransformation. Metabolites must be identifed by use of methodology such as gas chromatography coupled with mass spectroscopy and chemical synthesis. An assay method for detection of each major metabolite may be requested by the Agency.

(4) *Calculations.* (i) *Rate of absorption.* The rate of test substance absorption must be determined from plasma levels of the test substance plus metabolites (e.g., from total ^{14}C) for each animal in groups A, B, C, and D at a series of time intervals (e.g., 0.25, 0.5, 1, 2, 4, 6, and 8 hours) after dosing with radioactively labeled compound. Means and standard errors must be calculated for groups A, B, C, and D.

(ii) *Extent of absorption.* The extent of test substance absorption must be determined from the ratio of total excreted urinary or fecal radioactivity at each of several time periods after dosing:

Percent absorbed = total excreted urinary radioactivity (group B or C or D)/total excreted urinary radioactivity (group A) × 100 or Percent absorbed = total excreted fecal radioactivity (group B or C or D)/total excreted fecal radioactivity (group A) × 100. Calculations of this kind must be made from data obtained at several

intervals, such as 24, 48, 72, 96, 120, 144, and 168 hours. In order to obtain mean values and standard errors, these calculations must be made for individual animals in groups B, C, and D (numerator), using mean values for group A (denominator). Using standard statistics, these determinations for groups B and D will indicate whether or not absorption is dose dependent.

(iii) *Tissue binding.* Determinations as to whether the test substance and/or its metabolites bind covalently to tissues must be made for all groups by comparison of data from paragraph (f)(3)(i)(B) with data from paragraph (f)(3)(i)(C), each of this section.

(g) *Data reporting and evaluation.* (1) *Reporting.* The study must be reported in detail. Raw data may be contained in appendixes. In addition to information meeting the general reporting requirements of § 772.100–2(b)(8), Subpart A, the following data derived from tests on animals in all groups (described in paragraph (f) of this section) must be reported:

(i) Levels of radioactivity, together with percent recovery of the administered dose, in plasma, excreta, and the following tissues and organs of animals in all groups: Bone, brain, fat, gonads, heart, kidney, liver, muscle, spleen, tissues which displayed pathology, and residual carcass;

(ii) A semilogarithmic plot of plasma test substance concentration (expressed as nanogram equivalents of test substance/ml, as calculated from the specific activity of the test substance versus time, including a calculation of plasma t½ for all groups;

(iii) Percent absorption, by groups B, C, and D, determined from urinary or fecal radioactivity;

(iv) A full description of the sensitivity and precision of all procedures used to produce the data; and

(v) Information on the specific radioactivity of the test substance and site(s) of labeling.

(vi) Counting efficiency data must be made available to the Agency upon request.

(2) *Evaluation.* An evaluation of the data produced by this study must be submitted. The following points may be helpful in developing this evaluation.

(i) Plotting test substance plasma concentration (logarithmically) against time (arithmetically) will demonstrate the phases of absorption, tissue distribution (alpha), and excretion (beta) after test substance administration.

(ii) The curve representing intravenous test substance dosing will show only the tissue distribution and excretion phases. The data points can be used to calculate the half-times of tissue distribution (alpha) and excretion (beta). The calculations can be done by linear regression; estimates can be made graphically by the "feathering" method.

(iii) The urinary excretion data can be used similarly to calculate the half-time of the beta phase, following the pattern explained in paragraph (g)(2)(i) of this section.

(iv) The beta-phase t½ values will indicate clearly the potential of the test substance for bioaccumulation. Specific sites of bioaccumulation will be evident from the tissue distribution data.

(v) An analog computer can be used to prepare graphical plots of the probable plasma patterns after multiple dosing. A series of such plots can be prepared from data of groups B, C, and D. These plots will predict the steady-state plasma levels and the time necessary to attain them.

(vi) Predictions from group B data must be considered to be confirmed when group C data are not significantly different from group B data, with respect to peak plasma level; time at which peak plasma level occurred; t½ for the alpha phase; and t½ for the beta phase.

(vii) In addition to the material required above, the report should include a discussion section, in which the authors indicate clearly how they relate the metabolic and pharmacokinetic data to toxicologic observations in animals, and also to risks, if any, to human health. When the authors conclude that these relationships cannot be made on the basis of existing data, they should meet with Agency personnel to decide upon the design of additional studies.

(e) *Additional metabolic studies.* Additional, more specific studies may be required to clarify important points. Some areas for possible further study include: Binding by macromolecules in the blood, liver, gonads, and other tissues; placental transfer; entrance into breast milk; biotransformation by specific organs or tissues; absorption by dermal or inhalation routes of exposure; and measurement of levels of the test substance and its metabolites in the gonads. Plasma binding studies can be conducted, usually, *in vitro* with plasma. Placental transfer of a test substance can be determined readily by dosing pregnant rodents with radioactively labeled test substance and assaying their fetuses for radioactivity. Entrance of a test substance into breast milk can be determined similarly in rodents. Biotransformation studies can be performed, usually, with whole liver homogenates or with liver fractions such as microsomes or cytosol.

Appendix

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(3) Eichler, O., A. Farah, H. Herken and A.D. Welch. 1976. Handbook of Experimental Pharmacology. Springer-Verlag: New York. 377 pp.

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